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Pathogenesis of Human Enterovirulent Bacteria: Lessons from Cultured, Fully Differentiated Human Colon Cancer Cell Lines

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SUMMARY

Hosts are protected from attack by potentially harmful enteric microorganisms, viruses, and parasites by the polarized fully differentiated epithelial cells that make up the epithelium, providing a physical and functional barrier. Enterovirulent bacteria interact with the epithelial polarized cells lining the intestinal barrier, and some invade the cells. A better understanding of the cross talk between enterovirulent bacteria and the polarized intestinal cells has resulted in the identification of essential enterovirulent bacterial structures and virulence gene products playing pivotal roles in pathogenesis. Cultured animal cell lines and cultured human nonintestinal, undifferentiated epithelial cells have been extensively used for understanding the mechanisms by which some human enterovirulent bacteria induce intestinal disorders. Human colon carcinoma cell lines which are able to express in culture the functional and structural characteristics of mature enterocytes and goblet cells have been established, mimicking structurally and functionally an intestinal epithelial barrier. Moreover, Caco-2-derived M-like cells have been established, mimicking the bacterial capture property of M cells of Peyer's patches. This review intends to analyze the cellular and molecular mechanisms of pathogenesis of human enterovirulent bacteria observed in infected cultured human colon carcinoma enterocyte-like HT-29 subpopulations, enterocyte-like Caco-2 and clone cells, the colonic T84 cell line, HT-29 mucus-secreting cell subpopulations, and Caco-2-derived M-like cells, including cell association, cell entry, intracellular lifestyle, structural lesions at the brush border, functional lesions in enterocytes and goblet cells, functional and structural lesions at the junctional domain, and host cellular defense responses.

INTRODUCTION

The intestine is divided into four anatomical segments: the duodenum, jejunum, ileum, and colon (1). The intestinal epithelium functions as a physical and chemical barrier that protects the host from attack by potentially harmful enterovirulent microorganisms (2) (Fig. 1). To enable it to do this, the intestinal tract consists of a complex ecosystem that combines cells of various phenotypes lining the epithelial barrier plus the resident microbiota (Fig. 1 and 2). The intestinal mucosa has a surface coating of mucus that is secreted by the specialized goblet cells, which are also known as mucin-secreting cells, and which creates a physical barrier (3). Host defense systems against the unwelcome intrusion of pathogenic enteric microorganisms include both adaptive immunity and innate immunity. The intestinal epithelium senses the microbial environment in order to trigger strong cellular defense responses when this is required, by releasing host cell signaling molecules, such as cytokines and chemokines, which in turn trigger the recruitment of leukocytes and initiate the attraction of immune cells (4–7). Following infection by some enterovirulent bacteria, the host engages a rapid and appropriate innate immune response to control the enteric infection, but strong innate immune responses can be deleterious for the host by inducing severe lesions at the intestinal epithelial barrier. Toll-like receptors (TLRs) are one of the families of pathogen recognition receptors (PRRs), including retinoic acid-inducible gene 1 (RIG-1)-like receptors (RLRs), NOD-like receptors (NLRs), and DNA receptors (cytosolic sensors for DNA), which are known to play a crucial role in host defense. These PPRs recognize pathogens that express several signature molecules, known as pathogen-associated molecular patterns (PAMPs). After recognition by PAMPs, PRRs rapidly trigger an array of antimicrobial immune responses but also long-lasting adaptive immunity responses. The epithelium also provides antimicrobial peptides (AMPs), including defensins, C-type lectins, and cathelicidins produced by enterocytes and Paneth cells, all of which function to rapidly kill or inactivate pathogenic microorganisms (8, 9). In addition, autophagy, an evolutionarily conserved process by which cell constituents are broken down and recycled (10), also acts as a cell-autonomous defense against intracellular pathogenic bacteria (11, 12). Recently, overlaps between autophagy and innate immune signaling have been demonstrated, including responses to intracellular pathogens and damage-associated molecular patterns, such as the DNA-binding nuclear protein, high-mobility group box 1 (HMGB1), and interleukin-1β (IL-1β), TLRs, NLRs, and RLRs (13). Interestingly, the autophagic adaptors called SLRs (sequestosome 1/p62-like receptors) can be considered a new class of PRRs, contributing to autophagic control of intracellular microbes, including *Salmonella*, *Listeria*, and *Shigella* (13).

The adult human intestine contains trillions of microbes rep-

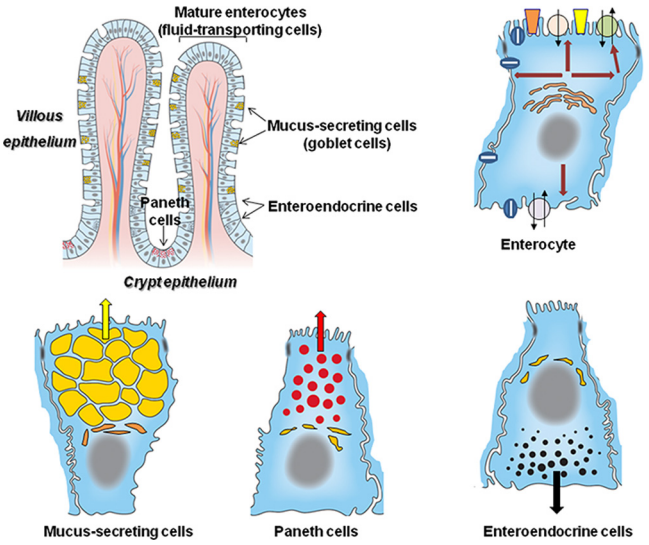

FIG 1 Intestinal epithelial barrier. The intestinal epithelium consists of a single layer of five phenotypes of highly polarized epithelial cells located at the crypts and villi. The crypts contain single progenitor stem cells which divide and differentiate in each intestinal cell phenotype during the crypt-villous cell migration, which is responsible for epithelial cell renewal after cell death and shedding of mature epithelial cells at the tip of the villus. Enterocytes, which constitute the most abundant intestinal cell phenotype, express a dense brush border composed of well-ordered microvilli facing the luminal compartment, in which are located hydrolases and transporters involved in absorption and secretion transcellular transporters, whereas others transporters are specifically localized at the lateral or basal membrane domains. Goblet cells produce brush border membrane-bound mucins and secreted mucins intracellularly packaged with large vesicles (yellow vesicles) and which after exocytosis into the luminal compartment form a thick mucus layer overlying the epithelium. Paneth cells located at the crypt of intestinal villi contain vesicles containing antimicrobial peptides and proteins (red vesicles), which after secretion into the luminal compartment exert a bactericidal effect against enteric bacterial pathogens. Enteroendocrine cells express intracellular secretory granules containing hormones and peptides (black vesicles), which after exocytosis into the interstitial space at the basal cell domain exert paracrine and endocrine functions. Yellow, red, and black arrows indicate the vectorial exocytosis processes of vesicles or granules. In enterocytes, brown arrows indicate the F-actin- or microtubule-dependent routes of intracellular traffic of cargo vesicles containing the functional proteins which are specifically vectorized to the apical, lateral, and basal domains of the polarized intestinal epithelial cells.

resenting hundreds of species and thousands of subspecies which have a predominantly symbiotic relationship with their host (14, 15). The microbial communities are segregated into three distinct clusters referred to as "enterotypes," each of which has a distinctive species and functional composition (16). The distribution of bacteria depends on the intestinal site; the duodenum contains far fewer resident bacteria than the ileum, and the colon contains a high level of anaerobic resident bacteria. The intestinal microbiota is involved in the regulation of intestinal epithelial cell turnover, in the structural and functional maturation of the epithelial barrier, in the first line of host defenses against the unwelcome intrusion of pathogenic bacteria, and in the immune modulation that plays a significant role in maintaining intestinal immune homeostasis (17, 18).

The mucosal surface of the intestinal tract is the largest body surface in contact with the external environment (200 to 300 m^2), and it is lined by a simple columnar epithelium that is folded to form a number of invaginations that increase the overall surface area (Fig. 1). The intestinal mucosa is formed by a single layer of columnar epithelial cells, the connective tissue of the lamina propria, and the muscularis mucosa (1). The epithelial intestinal cells are constantly regenerated from a source of multipotent stem cells located in the crypts of Lieberkühnn (19) (Fig. 1). The intestinal epithelium is completely renewed every 4 to 8 days by shedding mature epithelial cells at the tip of the villi as the result of a programmed cell death process known as "anoikis," which occurs when the cell is detached from the correct extracellular matrix, thus disrupting integrin ligation at the lateral and basal domains (20, 21). Four highly specialized cell phenotypes are present in the intestinal epithelium: enterocytes (also known as fluid-transporting cells), neuroendocrine cells, mucus-secreting cells (also known as goblet cells), and Paneth cells (Fig. 1). Moreover, M cells are located in the intestinal epithelium overlying mucosa-associated lymphoid tissues such as Peyer's patches (PPs), where they act as the antigen-sampling cells of the mucosal immune system and play a pivotal role in the pathogenesis of several enterovirulent microorganisms (22). During the crypt-to-villous migration, intestinal epithelial cells acquire a high degree of structural and functional polarization and form three selective membrane domains: the apical domain, which expresses a brush border facing the luminal compartment; the lateral domain, which functions as an epithelial cell-to-cell junctional domain establishing tight contacts with neighboring cells and sealing the intestinal epithelial barrier by the presence of three well-defined cell domains (the tight junction [TJ], the adherens junction [AJ], and the desmosome); and the basal domain, which connects the polarized epithelial cells to the basement membrane (23).

In the enterocytes, which are the major epithelial lineage of the intestine, the regulated sorting and surface delivery of apical and basolateral proteins lead to the selective presence of functional proteins at each membrane domains (Fig. 1). At the enterocytic brush border are present, for example, sucrase-isomaltase (SI), alkaline phosphatase (AP), lactase-phlorizin hydrolase, maltase-glucoamylase aminopeptidase N (APN), dipeptidylpeptidase IV (DPP IV), angiotensin I-converting enzyme, α-glucosidase, *p*-aminobenzoic acid peptide hydrolase, glycophosphatidylinositol (GPI)-anchored proteins, sodium/glucose cotransporter 1 (SGLT1), the GLUT1, GLUT2, GLUT3, and GLUT5 hexose transporters, peptide transporter 1 (PEPT1), H^+-coupled dipeptide transporter, cholesteryl ester transfer protein, Na^+/H^+ exchanger (NHE) isoforms, the Cl/HCO_3 exchanger DRA (downregulated in adenoma), and some members of the aquaporin (AQP) water channel family, transporting water as well as glycerol and other solutes of small molecules (24, 25). At the basolateral domain are present, for example, Na^+-K^+ ATPase and diamine oxidase. Moreover, membrane-bound receptors, for example, epidermal growth factor (EGF) receptor, insulin-like growth factor-binding proteins (IGF-BP-2, IGF-BP-3, and IGF-BP-4), vasoactive intestinal peptide (VIP) receptor, protease-activated receptor 2 (PAR-2), and nuclear hormone receptor peroxisome proliferator-activated receptor (PPAR) gamma are present and control cellular functions.

The goblet cells produce and excrete 17 human mucin-type glycoproteins encoded by the *MUC* gene family members *MUC1*, *MUC2*, *MUC3A*, *MUC3B*, *MUC4*, *MUC5B*, *MUC5AC*, *MUC6* to *-8*, *MUC10* to *-13*, and *MUC15* to *-17* (HUGO/GNC; http://www.hugo-international.org). A cluster of four mucin genes (*MUC2*, *MUC5B*, *MUC5AC*, and *MUC6*) encodes secreted mucins. Eight

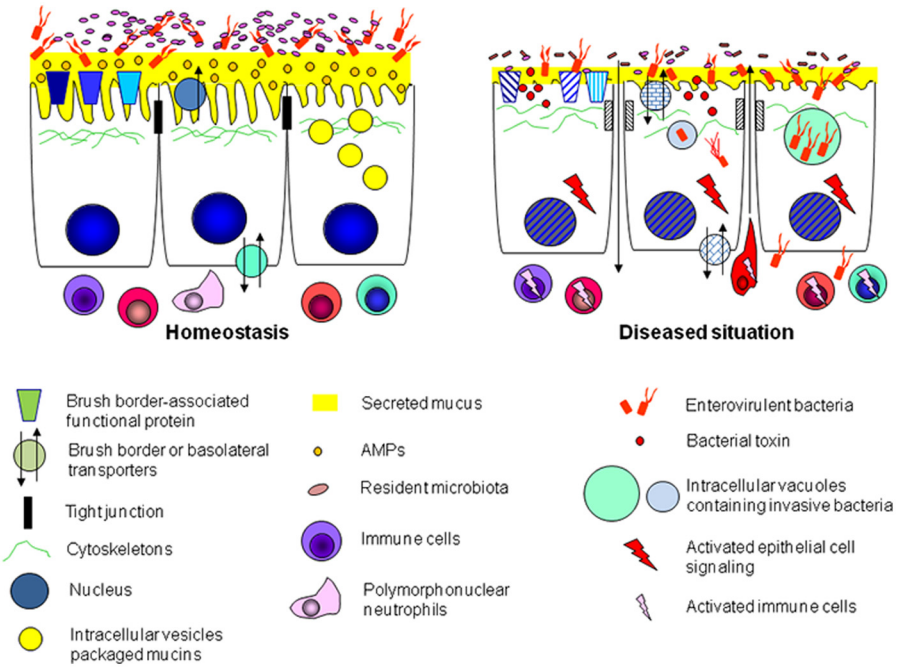

FIG 2 Overview of structural and functional mechanisms by which enterovirulent bacteria cause diarrhea. The intestinal epithelium consists of a single layer of highly polarized epithelial cells. The tight junction, a component of the apical junctional complex, seals the paracellular space between epithelial cells. Specific structural proteins compose the cytoskeleton and microtubule networks, which play pivotal roles in the polarized organization of intestinal cells. The brush border at the apical domain and basolateral cell domain contains proteins and transporters exerting specific intestinal functions. The intestinal epithelial barrier plays an essential role in maintaining immune homeostasis. The intestinal microbiota resides in the lumen, outside the mucus layer. Goblet cells secrete mucins which, combined with membrane-bound mucins, act as a physicochemical barrier and protect the epithelial cell surface. Antimicrobial peptides secreted by Paneth cells and enterocytes are localized within the mucus layer, forming the first chemical defense system against unwanted enteric pathogens. The lamina propria, located beneath the basement membrane, contains immune and dendritic cells. Enterovirulent bacteria use their adhesive factors to interact with the brush border membrane by hijacking membrane-bound molecules as receptors. Structural and functional brush border injuries results in adhesin/receptor interaction or T3SS-translocated bacterial effectors activating cell signaling pathways that lead to the cytoskeleton-dependent attachment/effacement (A/E) lesion of brush border microvilli or shedding of microvilli, which in turn results in the disappearance of brush border-associated proteins exerting specific intestinal functions. On the other hand, secreted cytotonic toxins, by binding to membrane-bound receptors, by endocytosis and retrograde traffic, or by T3SS-translocated bacterial effectors, activate signaling pathways for deregulating membrane-associated proteins controlling nutrient transport or functioning as ions and water channels. Moreover, secreted cytotoxic toxins after endocytosis induce cytoskeleton- or caspase-dependent cell death. In addition, via T3SS-translocated bacterial effectors or secreted toxins, enteric pathogens also target the junctional domain of polarized epithelial cells, inducing structural and functional lesions at the tight junctions and leading to a fault in the intestinal epithelial barrier. Invasive enterovirulent bacteria cross the epithelial cell membrane via a massive membrane rearrangement, penetrate into the host cells, and pursue sophisticated intracellular lifestyles within vacuoles containing bacteria. Other enteroinvasive bacteria, after escape from the vacuole, engage in actin-based movements within the cell cytoplasm for the penetration of neighboring cells via bacterium-induced transpodia. Adhering and invading enterovirulent bacteria trigger cellular defense responses, including, for example, the enhanced production/secretion of mucus and the production of proinflammatory cytokines and chemokines activating, in turn, immune cells of lamina propria. Moreover, some enterovirulent bacteria act to produce a loss of the first line of intestinal defenses by modifying the resident microbiota composition or altering the secretory process of mucus from goblet cells.

genes, *MUC1*, *MUC3A*, *MUC3B*, *MUC4*, *MUC12*, *MUC13*, *MUC16*, and *MUC17*, encode membrane-associated mucins. The intracellular processing of mucins involves synthesis, oligomerization in the endoplasmic reticulum, glycosylation in the *cis*- and *trans*-Golgi networks, and storage in granules (Fig. 1) (3, 26, 27). Intracellular small and large vesicles package the mucins, and the viscous mucus contained in vesicles is extruded after fusion of the vesicles and plasma membranes and the formation of a fusion pore through a process requiring an expulsive force (Fig. 1). This second pathway for mucin exocytosis involves the packaging and storage of mucins in large vesicles, from which their release is regulated by specific stimuli involving signaling molecules.

Paneth cells are pyramid-shaped, columnar exocrine cells (28). The ultrastructure of Paneth cells shows that they have a supranuclear region containing numerous highly electron-dense, apically located, eosinophilic secretory granules containing AMPs, and other antimicrobial molecules, including lysozyme, phospholipase A_2, and α_1-antitrypsin. AMPs are produced by Paneth cells and by enterocytes; all rapidly kill or inactivate pathogenic microorganisms (8).

Only about 1% of the epithelial cells lining the intestinal epithelium are enteroendocrine cells, which are subdivided into different cell types on the basis of their main secretory hormones and/or signaling molecules, such as ghrelin (B/D1 cells), serotonin (enterochromaffin cells), somatostatin (D cells), glucagon-like peptides (GLP-1 and GLP-2) and peptide YY (PYY) (L cells), gastrin (G cells), cholecystokinin (CCK) (I cells), secretin (S cells), glucose-dependent insulinotropic peptide (GIP) and xenin (K cells), motilin (M cells), and neurotensin (N cells) (29). Enteroendocrine cells are conical in shape without microvilli (closed cells) or with microvilli extending into the gut lumen (open cells). Intracellular secretory granules contain hormones and peptides that

are exocytosed in response to stimulation into the interstitial space from the basal cell domain (Fig. 1). Hormones and peptides act locally on neighboring cells (paracrine function), on neurons located in their proximity (neuronal function), or on distant targets, via the capillary networks of the lamina propria (endocrine function). Enteroendocrine cells secrete hormones and peptides that are well known for their effect on food intake and appetite, the regulation of glucose homeostasis, gut motility, and various other physiological functions.

The impermeability of the intestinal epithelium is structurally and functionally ensured by TJs, AJs, and the desmosome (23). TJs are the most apical intercellular protein complexes, formed by the interaction of transmembrane proteins claudins and occludin with the actin cytoskeleton via the zonula occludens 1 (ZO-1), ZO-2, and ZO-3 proteins. In addition, the transmembrane junctional adhesion molecule in the TJs is engaged in homophilic or heterophilic binding with other adhesion molecules such as integrins. Beneath the TJs, the multiprotein complexes forming the AJs are composed of transmembrane protein E-cadherin connected to intracellular components such as p120 catenin, β-catenin, and α-catenin linked to the actin cytoskeleton. Desmosomes localized beneath AJs are junctional complexes of transmembrane proteins, including desmoglein and desmocollin, which interact with desmoplakin linked to intermediate filaments. Functionally, TJs are responsible for sealing the intercellular space, and they act as a "gate" regulating the paracellular passage of particles and solutes, whereas the AJs and desmosomes act as adhesive bonds between intestinal epithelial cells, conferring mechanical strength on the intestinal epithelial barrier. Moreover, TJs also act as a "fence" separating the apical and basolateral membrane domains of polarized intestinal cells, thereby segregating the cell surface proteins and the lipids in each of the membrane domains. In addition, there is recent evidence that the enteric nervous system plays a role in modulating the epithelial barrier functions of the intestine (30).

Enterovirulent bacteria execute a prodigious array of complex functions in order to survive, multiply, and disseminate within host intestinal epithelial cells. To do this, genes coding for virulence factors are present in large clusters of virulence genes known as pathogenicity islands (PAIs), which are either present on plasmids or integrated into the chromosome. Each pathovar has developed specific mechanisms for attachment, hijacking, and subverting the host cell machinery. For example, interactions between virulence factors and host cell proteins activate host cell signaling pathways controlling the structural organization of the brush border cytoskeleton or regulating the polarized organization of intestinal epithelial cells. Moreover, enterovirulent bacteria target host cell signaling pathways regulating the intracellular traffic of functional proteins or the activities of membrane-associated functional proteins and transporters. In addition, host cell signaling pathways regulating the structural organization and the functions of the junctional domains of polarized intestinal epithelial cells are subverted by some enterovirulent bacteria, which in turn alters the barrier function of the intestinal epithelium. In this review, we analyze the molecular and cellular mechanisms of virulence developed by enterovirulent bacteria that have been identified using cultured, fully differentiated human colon carcinoma cell lines, subpopulations, and clone cells expressing the functional and structural characteristics of mature enterocytes, goblet cells, or M cells.

DIFFERENTIATED HUMAN COLON CANCER CELL LINES

Investigation of the mechanisms of pathogenicity of enterovirulent bacteria has long been complicated by the fact that human enterocytes and goblet cells are difficult to isolate and maintain for a long time in culture and tend to give rather variable results, depending on the particular donor. Since 1983, human colon carcinoma cell lines that are able to express the functional and structural characteristics of mature enterocytes or goblet cells in culture have been established (31), which has considerably facilitated the *in vitro* study of microbial pathogenesis. These cell lines were initially used to investigate basic questions related to the organization and functions of polarized human intestinal cells (32). When fully differentiated in culture, these cells display a polarized organization, form highly regulated junctional domains, and form a cell monolayer that physically and functionally mimics an intestinal epithelium barrier. They functionally mimic an intestinal epithelium barrier since each domain of these cells specifically expresses major differentiation-associated proteins that support specific intestinal functions. The parental fully differentiated human colon cancer cell lines and cell subpopulations and clones have been used since 1987 as models for investigating the cellular and molecular mechanisms by which enteric microbial pathogens hijack membrane-associated proteins, signaling pathways, and intracellular traffic functions, thus promoting structural and functional lesions and host cellular responses by enterocytes or mucin-secreting cells. An analysis of the literature reveals that some of the studies describing various different mechanisms of microbial enteric pathogenesis have been conducted using cultured human colon cancer cell lines that were not appropriately cultured. For examples, many studies have been conducted with parental HT-29 cells, which are wrongly described as "enterocyte-like cells"; in culture these cells formed multilayers of permanently undifferentiated cells which never expressed the polarized organization and the functional characteristics of fully differentiated epithelial cells that line the intestinal epithelium. For the parental Caco-2 cell line or clones, several studies indicating "enterocyte-like cells" have been conducted with cells cultured for 3 to 5 days, after which the cells either remain isolated or assemble to form islands of undifferentiated, nonpolarized cells. In other cases, Caco-2 cells have been cultured for 7 to 10 days, and even though they formed a confluent cell monolayer, the cells were in fact at an early state of cell differentiation in which the brush border was not well formed and the cells had not achieved functional differentiation. We therefore start this review by summarizing the structural and functional characteristics of human colon cancer cell lines that when cultured properly express the structural and functional characteristics of fully differentiated polarized epithelial cells lining the small intestine.

Differentiated Enterocyte-Like HT-29 Cell Subpopulations and Clones

In 1972, J. Fogh (Sloan Kettering Institute for Cancer Research, Rye, NY) (33, 34) isolated and cultured cells from human colonic adenocarcinomas. The parental colon cancer HT-29 cell line (33, 35) is composed mainly of undifferentiated cells, with a small minority of differentiated cells (~3 to 5% of total cells) (Table 1). Depending on the metabolic stresses exerted, such as glucose deprivation or its replacement by different substrates or culture in the presence of drugs, after an initial phase during which nearly all

TABLE 1 Characteristics of fully differentiated colon cancer cell lines, cell subpopulations, and clones[a]

Cell line, subpopulation, or clone	Reference(s)	Cell characteristics	Expressed intestinal functions
HT-29 Gal^+ and HT-29 Glc^-	36	Enterocyte-like cells forming in culture monolayers of polarized cells	Expression of a well-ordered brush border endowed by hydrolases such as SI, APN, DPP IV, and AP (36, 37)
HT-29 Rev $Glc^{-/+}$	38	Enterocyte-like cells forming in culture monolayers of polarized cells[b]	Expression of a well-ordered brush border endowed by hydrolases such as SI, APN, DPP IV, and AP (38, 42), ATPase activities (46), CEA (45), chloride secretion (47, 48), oligopeptide transport (49), Na^+/glucose cotransport (50); presence of mature junctional complexes (39); receptor for VIP (52, 53) and PAR2 (51); Presence of AMPs (54)
HT-29-D4, HT-29-D9	39		
HT29.74	40		
HT-29-18-C_1	41		
Clone HT-29.19E	42		
HT29 clone 13	43		
Clone HT-29.cl16E	42	Homogeneous subpopulations of goblet cells forming in culture monolayers of polarized cells producing mucins and secreting mucus[b]	Expression of brush border endowed by hydrolases such as SI, APN, and DPP IV (38); presence of MUC2, MUC5AC, and MUC6 secreted mucins and MUC1, MUC3, and MUC4 membrane-associated mucins (62–68); presence of intracellular vesicles containing mucus; presence of two secretory pathways of mucins, a constitutive pathway and a cell signaling-regulated pathway (27)
HT29-18N2 clone	41, 60		
HT29-MTX	38		
HT29-SB	59		
HT29-FU	61	Mixed population of enterocyte-like cells (90%) and randomly distributed, mucin-secreting cells (10%)[b]	Expression of brush border endowed by hydrolases such as SI and DPP IV (61); presence of MUC2 and MUC5AC secreted mucins and MUC4 membrane-associated mucin (61); presence of AMPs (54)
Caco-2	82	Spontaneously differentiate in culture in enterocyte-like cells forming monolayers of polarized cells[c]	Expression of brush border endowed by functional proteins such as SI, lactase, AP, APN, and DPP IV (69, 108), SGLT1, GLUT1, GLUT2, GLUT3, and GLUT5 (123), hPEPT1 (126), H^+-coupled dipeptide transporter (127), NHEs (128, 129), DRA (130), MCT1 (131), Na^+-K^+-ATPase (110), diamine oxidase (138), cholesteryl ester transfer protein (132), PAR2 (51), AQP3 and AQP10 (133–137), and receptors, including the epidermal growth factor receptor (139), the insulin-like, growth factor-binding proteins (IGF-BP-2, IGF-BP-3, and IGF-BP-4) (139), VIP receptor (52), and PPAR gamma (140); mechanisms of sorting and surface delivery of apical and basolateral functional proteins (103, 104); in-and-out efflux systems controlling the intestinal transport of drugs (141); positive TER in cell monolayers and expression of TJ proteins ZO-1, -2, and -3, occludin, and claudins claudin-1, -2, -3, -4, -12 but not claudin-5; formation of fluid-filled, blister-like domes resulting from both the paracellular and transcellular pathways of ion and water transport and which determine the net apical to basolateral vectorially transported water (142); presence of AMPs (54, 143)
Clones Caco-2BBe 1 and 2	86	Spontaneously differentiate in culture in enterocyte-like cells forming monolayers of polarized cells[c]	
Clone 40	83		
Clones Caco-2/1 to 16	84		
Clone cl1	88		
Clone Caco-2/AQ	89		
Clone NGI3	90		
Clones 1, 20, and 21	91		
26 clones from late or early passages, including Caco-2/TC7	94		
Caco/B7	92		
Clones NCL-1 to -12	93		
T84	153	Spontaneously differentiate in culture in polarized colonic cells[d]	Expression of brush border endowed by hydrolases such as SI, DPP IV, and CEACAMs; regulated chloride secretion (157), NHE-1, -2, and -4 (158), $Na^+/K^+/2Cl^-$ transport (159), chloride and HCO_3^- secretion (160), ENT1 and ENT2 (161), the CFTR chloride channel (162, 163), and production of exosomes (164); High level of positive TER, resulting in tightly formed and highly regulated functional TJs expressing ZO-1, occludin, and claudins (156); PMNL transmigration

[a] Abbreviations: SI, sucrase-isomaltase; APN, aminopeptidase N; DPP IV, dipeptidylpeptidase IV; AP, alkaline phosphatase; CEACAMs, carcinoembryonic antigen cell adhesion molecules; CEA, carcinoembryonic antigen; VIP, vasoactive intestinal peptide; PAR2, protease-activated receptor 2; PEPT1, H^+/epycotransport system; NHEs, Na^+/H^+ exchanger isoforms; DRA, downregulated in adenoma Cl/HCO_3 exchanger; CFTR, cystic fibrosis transmembrane conductance regulator chloride channel; LTP-I, cholesteryl ester transfer protein; PPAR, nuclear hormone receptor peroxisome proliferator-activated receptor (PPAR); SGLT1, GLUT1, GLUT2, GLUT3, and GLUT5, hexose transporters; ENT, Na^+-independent equilibrating nucleoside transporters; PMNLs, polymorphonuclear leukocytes. It is important that the cells are maintained in the laboratory under strict conditions of culture: low density of seeding (12×10^3 cells/cm^2), 6-day passage frequency, use of DMEM containing the appropriated glucose concentration (as indicated for each line) supplemented with appropriated percentage of heat-inactivated (56°C, 30 min) fetal bovine serum (as indicated for each line) and with or without nonessential amino acids (as indicated for each line), and culture in a 10% CO_2–90% air atmosphere. The medium was changed at 24 or 48 h.

[b] DMEM, 25 mM glucose, and 10% calf fetal serum at 37°C in a humidified 10% CO_2–90% air atmosphere for 21 days in culture.

[c] DMEM, 25 mM glucose, 20% inactivated fetal calf serum, and 1% nonessential amino acids at 37°C in a 10% CO_2–90% air atmosphere for 15 days in culture.

[d] A 1:1 (vol/vol) mixture of Dulbecco-Vogt modified Eagle medium and Ham's F-12 medium supplemented with 6% fetal calf serum (pH 7.5) at 37°C in a 10% CO_2–90% air atmosphere for 15 days in culture.

the undifferentiated cells die, the surviving cells that have resisted the stress consist of the small number of differentiated cells that were present at low levels in the parental HT-29 cell line. Several subpopulations of absorptive HT-29 cell subpopulations have been established (Table 1). The first enterocyte-like subpopulation of HT-29 cells, designated HT-29 Gal^+, was obtained by a selection culture process in which galactose replaces glucose in the culture medium (36). A second enterocyte-like subpopulation of

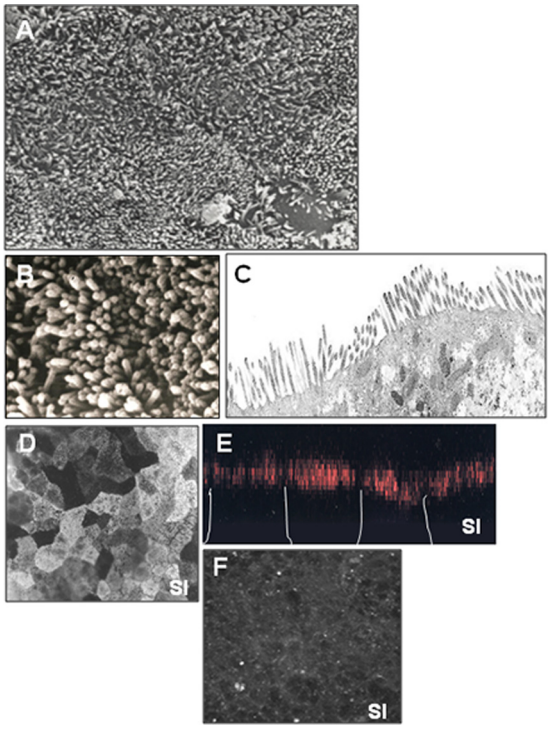

FIG 3 Fully differentiated human colon cancer HT-29 $Glc^{-/+}$ cell subpopulation expressing the structural and functional characteristics of mature enterocytes of the small intestine. (A and B) Scanning electron microscopy examination shows the presence of a dense and well-organized brush border. (C) Transmission electron microscopy examination shows the well-organized microvilli. (D) Confocal laser scanning microscopy examination of SI immunofluorescence labeling shows the mosaic pattern of expression (*x-y* section). (E) Confocal laser scanning microscopy examination of SI immunofluorescence labeling shows the specific expression of hydrolase at the apical domain (*x-z* section). (F) Lack of SI immunofluorescence labeling in confluent undifferentiated parental HT-29 cells. Confluent cells at 21 days in culture are shown.

HT-29 cells, designated HT-29 Glc^{-} cells, was established from the parental HT-29 cell line by glucose deprivation during culture (37). The enterocyte-like subpopulation designated HT-29 Rev $Glc^{-/+}$, which maintains the differentiation characteristics permanently at subconfluence, was established after switching HT-29 Rev Glc^{-} cells back into a standard glucose-containing medium (38) (Fig. 3). Other enterocyte-like cell subpopulations, designated HT-29-D4 (39), HT29.74 (40), and HT-29-18-C_1 (41), have also been established. From the parental HT-29 cell line, two permanently differentiated clonal cell lines composed of absorptive cells have been established and designated HT-29.19E, obtained after sodium butyrate treatment (42), and HT29 clone 13, obtained after adenosine deprivation using adenosine deaminase (43). All the enterocyte-like HT-29 cell subpopulations or cell clones expressed a well-ordered apical brush border expressing human functional proteins found in the enterocytes of the human small intestine, a tight junctional domain, and a basal domain (31) (Fig. 3). Polarized HT-29 cell subpopulations and clones have been used to investigate cell polarization (44) and expression of several differentiation-associated intestinal functional proteins, including SI, APN, DPP IV, and AP (36–38, 42), carcinoembryonic antigen (CEA) (45), ATPase activities (46), chloride secretion (47, 48), oligopeptide transport (49), Na^{+}/glucose cotransport (50), PAR2 (51), receptor for VIP (52, 53), and AMPs (54) (Table 1). They also exhibit a controlled production or cellular effects of cytokines such as interleukin-4 (IL-4) and IL-13 (55), tumor necrosis factor alpha (TNF-α) (56), IL-1 (57), and IL-8 (58). In contrast to the Caco-2 and T84 cell lines, these permanently fully differentiated, fluid-transporting HT-29 subpopulations have rarely been used to study the pathogenicity mechanisms of enteric bacteria.

Differentiated Mucin-Secreting HT-29 Cell Subpopulations and Clones

Depending on the culture selection system used, several mucus-secreting HT-29 subpopulations have been selected and mucus-secreting HT-29 cell clones have been established from the parental HT-29 cell line. The first permanently differentiated clonal cell line, HT-29.cl16E, was a homogeneous, mucin-secreting, intestinal cell population that emerged from the parental HT-29 cell line after sodium butyrate treatment (42). A second mucus-secreting, clonal derivative (HT29-SB) has also been established (59). Growing the parental HT-29 cell line in the absence of glucose results in the selection of homogeneous columnar cells with the typical goblet cell morphology, which have been designated the HT29-18N2 clone (41, 60). Adaptation of the parental HT-29 cell line to lethal concentrations of methotrexate (MTX) (38) and 5-fluorouracil (FU) (61) has been shown to result in the emergence of subpopulations of cells that are all stably committed to differentiation (38, 61). The HT29-MTX cell subpopulation is a homogeneous subpopulation of goblet cells that secretes the mucus and mucins of gastric and colonic immunoreactivity (Fig. 4A to G). In contrast, the cells in the HT29-FU cell subpopulation are all differentiated and exhibit 2 phenotypes: most are enterocyte-like cells, with a few randomly distributed mucin-secreting cells (61) (Fig. 4H and I). Both secreted and membrane-bound intestinal mucins have been characterized in HT29-MTX and HT29-FU cell subpopulations, since MUC2 and MUC4 were highly expressed in HT29-FU and MUC3 and MUC5AC were highly expressed in HT29-MTX (62–68). The mucin-secreting HT-29 cell clones and subpopulations have been used to investigate the regulation of mucus transport (69–78) and the functionality of human intestinal mucin-secreting cells (26, 27). Moreover, mucin-secreting HT-29 cell subpopulations and clones have been used to investigate the role of human mucins in bacterial pathogenesis (62, 63, 79–81). Enterocytic cells in fully differentiated HT29-FU cells expressed AMPs PR-39 and cecropin P1 (54).

In some pathogenic or commensal intestinal microbiota bacteria and parasites, the mucus gel can serve at least two functions. First, it can be a source of nutrients for bacterial growth, thus increasing the colonization of the intestine by the adhering bacteria, which have the ability to survive and multiply in the outer areas of the mucus layer. Second, the mucus coat overlying the microvillous surface contributes to the host defenses by preventing bacterial or parasite adhesion or invasion and the binding of toxins to the intestinal brush border.

Differentiated Enterocyte-Like Parental Caco-2 Cell Line and Clones

The parental colon cancer Caco-2 cells were established from passage 14 of the cell line obtained from J. Fogh (33, 34). The cells differentiate spontaneously in culture, characterized by the appearance of structural and functional properties of the enterocytes

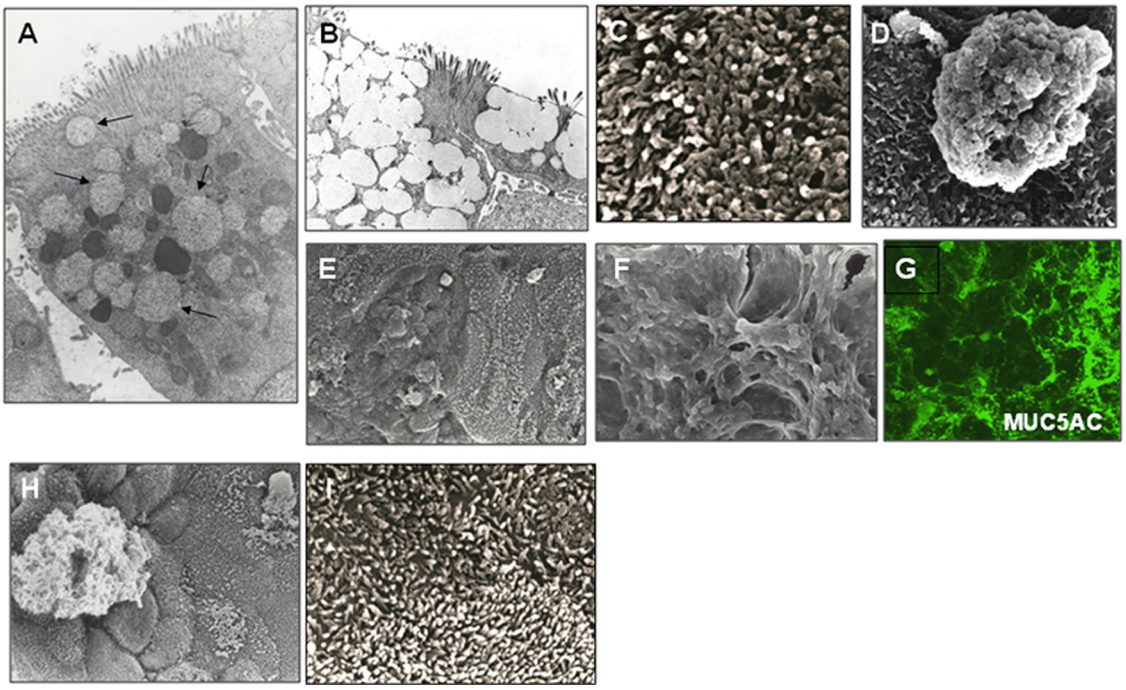

FIG 4 Fully differentiated mucus-secreting human colon cancer HT29-MTX and HT29-FU cell subpopulations expressing the structural and functional characteristics of intestinal mucus-secreting goblet cells. (A) Transmission electron microscopy examination of confluent fully differentiated HT29-MTX cells shows the polarized organization of the cells, the brush border, and the intracellular dense vesicles containing packaged mucins. (B) Transmission electron microscopy shows the intracellular vesicles containing packaged mucins near the apical domain. (C) Scanning electron microscopy examination shows the brush border of HT29-MTX cells after elimination of the mucus gel. (D) Scanning electron microscopy shows the secretion of mucus at the cell surface. (E and F) Scanning electron microscopy examination of HT29-MTX cells shows the dense mucus gel at the cell surface. (G) Confocal laser scanning microscopy examination shows the immunofluorescence labeling of secreted MUC5AC mucin in HT29-MTX cells (*x-y* section). (H) Scanning electron microscopy examination of postconfluent HT29-FU cells shows mucus secreted by a randomly distributed mucus-secreting HT29-FU cell and adjacent enterocyte-like HT29-FU cells. (I) High-magnification micrograph shows the dense and well-organized brush border in HT29-FU enterocyte-like cells. Confluent cells at 21 days in culture are shown. The arrows in A indicate the intracellular vesicles containing mucus.

of the small intestine (35, 82) (Fig. 5). Several clones of Caco-2 cells have been established: clone 40 (83), clones Caco-2/1 to Caco-2/16 (84), clones Caco-2BBe 1 and 2 (also referenced as C2BBe 1 and 2) (85–87), clone cl1 (88), clone Caco-2/AQ (89), clone NGI3 (90), clones 1, 20, and 21 (91), Caco/B7 (92), and clones NCL1 to -12 (93). Moreover, 26 Caco-2 clones have been isolated from early and late passages of parental Caco-2 cells, 8 clones from passage 29 and 18 clones from passage 198, including the most commonly used Caco-2/TC7 clone (94). Importantly, it should be noted that the fully differentiated Caco-2 cells and Caco-2 clones, although they are of colonic origin, express apical and basolateral proteins and display the specific functions of the mature enterocytes of the small intestine (31). In culture, the fully differentiated parental Caco-2 cell line and its clones form a cell monolayer that mimics the intestinal epithelial barrier (Fig. 5B). The differentiation process in the culture of Caco-2 cells is growth related; exponentially dividing cells are undifferentiated, and differentiation starts at confluence when the cells stop dividing (Fig. 5I) and closely mimics the differentiation of enterocytes that occurs along the crypt-villus axis of the small intestine.

Parental Caco-2 cells and clones provide the best model for investigating the cell polarization that develops after confluence in culture and the roles of structural proteins, such as actin, fodrin, fimbrin, villin, myosin I and II, and plastin-1, in the structural and functional brush border assembly (Fig. 5B to I) (85–87, 95, 96). For intestinal epithelial cell polarity to develop, there is a complex sorting and cargo-trafficking machinery that carries out the intracellular transport of some functional proteins so that they can be directly and efficiently targeted to the basolateral membrane, whereas the apical functional proteins reach the apical microvillous membrane by several different routes (97). Some proteins are directly targeted to the apical microvillous membrane, whereas others are addressed to the microvillous membrane after a transitional stage in the lateral domain. In addition, a subapical cell compartment seems to function as a docking platform for vesicles containing functional proteins (98). Parental Caco-2 cells and clones have also been used to identify the mechanisms underlying the sorting and surface delivery of apical and basolateral proteins in human enterocytes (99–107) and to find out how functional intestinal proteins take their place in cell membrane domains, including brush border-associated functional proteins such as SI (82, 84, 86, 90, 94, 100, 106, 108–114), AP (110, 111), lactase-phlorizin hydrolase (108, 115), maltase-glucoamylase (108), APN (90, 108, 111), DPP IV (82, 90, 100, 108, 116–121), angiotensin I-converting enzyme (108), α-glucosidase (122), *p*-aminobenzoic acid peptide hydrolase (108), SGLT1, GLUT1, GLUT2, GLUT3, and GLUT5 (123–125), PEPT1 (126), H^+-coupled dipeptide transporter (127), NHEs (128, 129), Cl/HCO_3 exchanger DRA (130), monocarboxylate transporter 1 (MCT1) (131), cholesteryl ester transfer protein (132), AQP3 and AQP10 (133–137), Na^+-K^+ATPase (110), and diamine oxidase (117, 138) (Table 1) (Fig. 5G to I). Parental cells and clones have been also used to

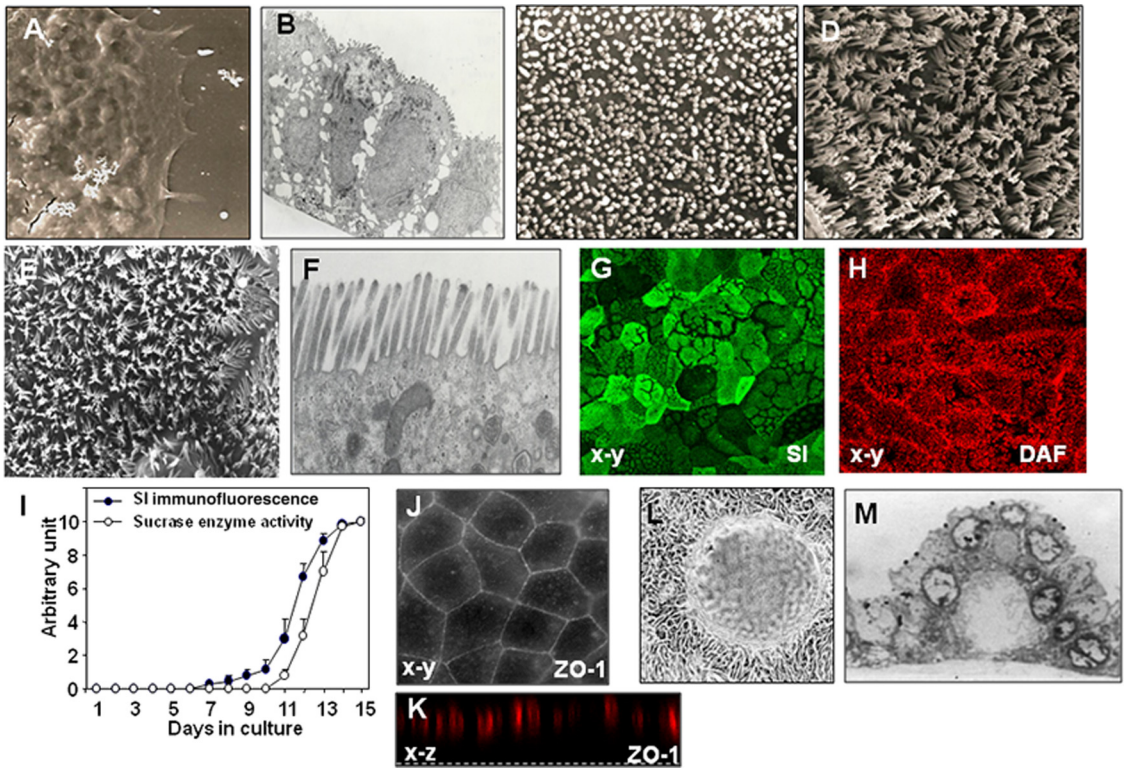

FIG 5 Human colon cancer Caco-2/TC7 clone cells expressing the structural and functional characteristics of mature enterocytes of the small intestine. Scanning electron microscopy micrographs show an undifferentiated cell at 3 days in culture (A), the short microvilli at the dense brush border in postconfluent cells at 10 days in culture (C), and the well-organized and dense brush border in postconfluent cells at 15 days in culture (D and E). (B) Transmission electron microscopy micrograph shows the polarized organization of postconfluent cells forming a monolayer at 15 days in culture. (F) Transmission electron microscopy micrograph shows the well-ordered brush border microvilli in postconfluent cells at 15 days in culture. (G and H) Confocal laser scanning microscopy examination shows the immunofluorescent labeling of two brush border-associated functional proteins (*x-y* section). (G) Mosaic pattern of expression of sucrase-isomaltase (SI). (H) Typical punctuate distribution of the glycophosphatidylinositol-anchored glycoprotein decay-accelerating factor (DAF). (I) Increase in expression of SI immunofluorescence labeling and sucrase enzyme activity as a function of days in culture. (J and K) Confocal laser scanning microscopy examination shows the immunofluorescent labeling of TJ-associated ZO-1 localizing at the cell-to-cell contact of the cell monolayer (*x-y* section in panel J and *x-z* section in panel K). (L and M) Confocal laser phase-contrast microscopy micrograph (L) and transmission electron microscopy micrograph (M) showing a fluid-forming dome in the cell monolayer.

study the regulatory functions of membrane-bound receptors, including EGF receptor (139), IGF-BP-2, IGF-BP-3, and IGF-BP-4 (139), VIP receptor (52), PAR2 (51), and PPAR gamma (140). Moreover, Caco-2 cells and clones have been used to investigate the in-and-out efflux systems controlling the intestinal transport of drugs (141).

Parental Caco-2 cells and clones forming a monolayer at the fully differentiated state expressed a well-organized and regulated cell-to-cell junctional domain including TJs formed by ZO-1, -2, and -3, occludin, and claudin-1, -2, -3, -4, -12, but not claudin-5 (Fig. 5B, J, and K). In addition, the fully differentiated Caco-2 cell monolayer forms fluid-filled, blister-like domes that are highly dynamic structures formed as the result of both the paracellular and transcellular pathways of ion and water transport and which determine the net apical-to-basolateral vectorially transported water (142) (Fig. 5L and M). Consistent with the production of AMPs by enterocytes (8, 9), parental fully differentiated Caco-2 cells express human cathelicidin LL-37/human cationic antimicrobial protein 18 (LL-37/hCAP18) mRNA and protein and showed positive immunoreactivity for lysozyme, α1-antitrypsin, and AMPs PR-39 and cecropin P1, whereas fully differentiated T84 cells do not (54, 143). The parental fully differentiated Caco-2 cell line and clones have been extensively used to investigate the cellular and molecular mechanisms by which human enterovirulent bacteria and enteric viruses create structural and functional cellular lesions and trigger cellular immune responses in the intestinal barrier. Moreover, fully differentiated Caco-2 cells and their clones have been used to investigate how bacterial species in the human intestinal microbiota control the pathogenesis of human enteropathogens.

Parental Caco-2 cells are known to be a homogeneous enterocyte-like cell line, but surprisingly, several reports have documented the presence of *MUC* genes and MUC proteins, including the *MUC1F* gene (144) and MUC1 mucin (145–148), the *MUC2* gene (145), the *MUC3A* and *MUC3B* genes (145, 149), the *MUC4* gene (150) and MUC4 mucin (151), and the *MUC5B* gene (152) and MUC5B mucin (148). Interestingly, the fully differentiated NCL2 clone recently isolated from the parental Caco-2 cell line shows the homogeneous presence of cells apically secreting a glycocalyx-like or mucin-like material (93).

Differentiated Colon Crypt T84 Cell Line

The T84 cell line is composed of colonic epithelial cells derived from a human colonic carcinoma (153). The cells were grown to

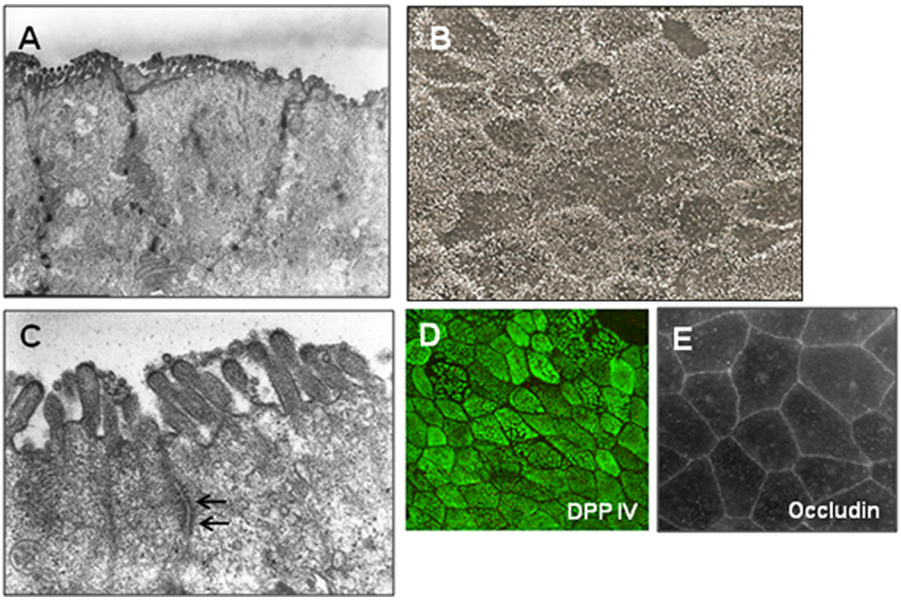

FIG 6 Fully differentiated human colon cancer T84 cell line expressing the structural and functional characteristics of colonic cells. (A) Scanning electron microscopy micrograph shows the well-organized brush border with short microvilli. (B and C) Transmission electron microscopy micrograph shows the polarized organization of the cells forming a monolayer and the apical brush border composed of short microvilli. Arrows indicate TJs. (D) Confocal laser scanning microscopy micrograph shows the mosaic pattern of expression of immunolabeled functional brush border-associated dipeptidyl-peptidase IV (DPP IV) (*x-y* section). (E) Confocal laser scanning microscopy examination of immunofluorescence labeling of TJ-associated occludin protein shows the expression at the cell-to-cell contact of confluent cells (*x-y* section). Confluent cells at 15 days in culture are shown. (Panels A and C courtesy of P. Hofman, reproduced with permission.)

confluence as a monolayer attached to the surface of the culture dish or permeable supports. These cells were highly polarized (Fig. 6A), with short microvilli on the apical membrane facing the medium (Fig. 6B and C), a basolateral membrane, and TJs and desmosomes between adjacent cells (Fig. 6C). Fully differentiated cells expressed human functional intestinal proteins at the brush border (Fig. 6D) and structural and functional proteins regulating paracellular passage at the TJs (Fig. 6E) (Table 1). Mounted in Ussing chambers, this cell line provides an excellent model system for studying electrolyte transport processes and the functions of voltage-dependent channels by electrical circuit analysis and membrane-associated vectorial cell transport systems (154–156). Fully differentiated T84 cells have been used to investigate the regulation of chloride secretion (157), NHE-1, -3, and -4 (158), $Na^+/K^+/2Cl^-$ transport (159), chloride and HCO_3^- secretion (160), Na^+-independent equilibrating nucleoside transporters ENT1 and ENT2 (161), the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel (162, 163), and production of exosomes (164). Since they express a high level of transepithelial electrical resistance (TER) (154–156), T84 cells provide the best model to investigate the impact of enterovirulent bacterial pathogens and enteric viruses and bacterial toxins on the structural organization of TJs and the transport functions of human colonic cells. The T84 cell line offers the best model for investigating how enterovirulent pathogens induce the transmigration of polymorphonuclear leukocytes (PMNLs) across the intestinal barrier and the resulting cellular consequences (165, 166). The regulation of the PMNL transmigration across T84 cell monolayer has been described in detail (167–178). Moreover, several studies have analyzed the consequences of the transmigration of PMNLs for the polarized cells (179–182) and the changes seen in the PMNLs themselves (183–187).

The presence of the *MUC1*, *MUC2*, *MUC3*, and *MUC5AC* genes (188–192) and the regulation of *MUC* gene expression and the production or secretion of mucins in T84 cells (190–198) have been described. It is noteworthy that unlike the case for the HT-29 and Caco-2 cell lines, no clone or subpopulation has so far been established from the parental T84 cell line.

Parental Nondifferentiated Ileocecal Adenocarcinoma HCT-8 Cell Line and Subpopulations of Partially Differentiated HCT-8 and Fully Differentiated HCT8-MTX Cells

The parental human ileocecal adenocarcinoma HCT-8 cell line (199) and variants (HCT-8, HCT-8R, HCT-8 Nu 1, HCT-8R Nu 1, HCT-8 Nu 2, and HCT-8R Nu 2) (200) express the CEA as fully differentiated HT-29 cell subpopulations and clones and as parental fully differentiated Caco-2 cells. Parental HCT-8 cells cultured at postconfluence (in Eagle's minimal essential medium supplemented with 2 mM glutamine and 10% fecal calf serum or in Dulbecco modified Eagle's medium [DMEM] [25 mM glucose] supplemented with 10% inactivated fetal calf serum and cultured at 37°C in a 10% CO_2–90% air atmosphere for 21 days in culture with culture medium changed daily) form a heterogeneous layer with large clusters of piled-up, flat cells and rounded cells alternating with small areas of more spread-out cells in which the cells are attached to each other by poorly organized junctional complexes (Fig. 7A to C) (201). Most of the cells were not differentiated, since villin and DPP IV were found at levels corresponding to unpolarized membrane expression, but a small proportion of the isolated cells or of the cells in clusters exhibited poorly organized cell extensions. Moreover, it has been found that ZO-1 is distributed scantly and anarchically in these HCT-8 cells organized in clusters. When cultured on permeable matrices and at late postconfluence stages, parental nondifferentiated HCT-8 cell layers

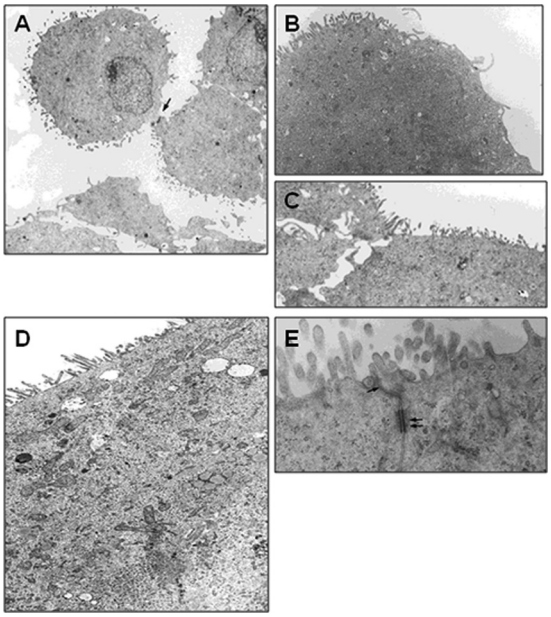

FIG 7 The parental nondifferentiated ileocecal adenocarcinoma HCT-8 cell line and fully differentiated HCT8-MTX cells. (A to C) Transmission electron microscopy examination of parental HCT-8 cells shows the cells expressing unorganized cell extensions at the periphery of isolated cells (A and B) and the presence of no well-ordered microvillus-like structures in cells localized at the periphery of cell clusters (C). (D and E) Transmission electron microscopy examination of fully differentiated HCT8-MTX cells expressing a short and well-ordered brush border. Cells were observed after 15 days in culture. (Reprinted from reference 201 with permission of the publisher.)

displayed a low TER (202, 203). HCT-8 variants, such as HCT-8R cells, appeared to be entirely pleomorphic, and colonies contained fusiform, epithelial-like cells without junctional complexes and microvilli that appeared to be irregular in length, shape, and spacing. Parental HCT-8 cells have been grown in microgravity using a rotating-wall vessel (RWV) apparatus (204). After 10 days in culture, layered HCT-8 cell aggregates (HCT-8 organoids) developed, showing a villus-like cell organization. Interestingly, the cells in the HCT-8 organoids displayed a shape characteristic of polarized cells, indicating greater cellular differentiation than conventionally grown HCT-8 cells. Moreover, the cells in the upper part of the HCT-8 organoids developed short and not well-organized microvilli, unlike those observed in enterocyte-like parental Caco-2 cells and clones and HT-29 subpopulations and clones but resembling those present in T84 cells. Consistent with the better polarization and the presence of sparse microvilli, the cells localizing in the upper part of HCT-8 organoids apically expressed villin and expressed the TJ-associated ZO-1 protein in a more organized fashion than the parental HCT-8 cells. Parental HCT-8 cells have been used mainly to investigate drug cytotoxicity, resistance to antitumor agents, and the deregulated functions in cancer cells. Despite the fact that parental HCT-8 cells do not display the structural and functional characteristics of enterocytes, they have been used to study the interactions of a small number of enterovirulent bacteria with human intestinal epithelial cells (205–216) and bacterial toxin effects (217–219).

An HCT-8 cell subpopulation (HCT8-MTX) has been obtained by culturing these cells in the presence of MTX (201). In contrast to parental nondifferentiated HCT-8 cells, HCT8-MTX cells formed a homogeneous cell monolayer of polarized cells expressing TJs, with short and ordered microvilli forming a brush border at the apical cell surface (Fig. 7D and E) and with a linear expression of villin, DPP IV, CEA and MUC1 corresponding to expression at a polarized membrane. Moreover, HCT8-MTX cells, like fully differentiated Caco-2 cells, formed a number of domes which are indicative of a cell monolayer with vectorial ion transport properties.

Coculture Models

Peyer's patches (PPs), also known as follicle-associated epithelium, are areas in the mammalian gut mucosa consisting of aggregated and isolated lymphoid follicles separated from the intestinal lumen by a single layer of columnar epithelial cells. In these randomly distributed and particular intestinal cell areas, a relatively small number of highly specialized epithelial cells, known as M (microfold or membranous) cells, have been identified (22, 220). The phenotype of human M cells has long remained elusive due to the difficulty of isolating these sparsely occurring intestinal cell areas. M cells play a central role in initiating mucosal immune responses by transporting intact foreign antigens and commensal bacteria into the underlying lymphoid tissue. In this way, the mucosal immune system encounters the virtually limitless variety of antigens that enter the body through the gut mucosa and reacts by mounting specific mucosal and systemic immune responses. M cells are also major adhesion and invasion sites for several invasive enteric pathogens (221). Moreover, after crossing the follicle-associated epithelium, the invading bacteria face phagocytic cells, including the macrophages that are present in the follicle dome. For several enteroinvasive pathogens, the outcome of infection depends on the capacity to survive in the presence of macrophages or within macrophages after phagocytosis and how these invasive species do this (222).

A model of M-like cells has been obtained by coculturing freshly isolated BALB/c mouse PP lymphocytes with parental fully differentiated Caco-2 cells, triggering the phenotypic conversion of Caco-2 cells into cells that express the morphological and functional properties of M cells (223, 224). A more stable model of M-like cells has been obtained by coculturing fully differentiated parental Caco-2 cells (225) or Caco-2 cl1 clone cells (226) with the human Burkitt's lymphoma Raji B cell line. Another model has been constituted from fully differentiated Caco-2 cl1 clone cells cultured in the presence of freshly isolated human blood lymphocytes (227). The mechanism(s) by which the fully differentiated Caco-2 cells are converted into M cell-like cells has not yet been entirely elucidated. Caco-2 cl1 cells cocultured with mouse PP lymphocytes display much lower levels of SI in their apical membranes than their differentiated Caco-2cl1 counterparts (223); a process that could account for this has been proposed. The conversion of M cell-like cells may be due to the disruption of the brush border resulting from a mouse PP lymphocyte-triggered dedifferentiation process (226). Consistently with this, there was an ~2-fold downregulation of the SI promoter in the M cell-like cells compared to Caco-2 cl1 cells, indicating that the lymphoepithelium-induced downregulation of the differentiation- and brush border-associated SI in converted M cell-like cells is the result of a lymphoepithelium-triggered dedifferentiation process of fully differentiated Caco-2 cl1 cells (226). The dedifferentiation of Caco-2 cl1 cells, converting them into M cell-like cells, probably results from a contact-dependent mechanism that occurs when

mouse PP lymphocytes come into contact with fully differentiated Caco-2 cl1 cells, rather than as a result of the action of molecules secreted by mouse PP lymphocytes. Indeed, after coculture with mouse PP lymphocytes, all the fully differentiated Caco-2 cl1 cells were not converted into M cell-like cells, and only randomly distributed M cell-like cells were observed within the Caco-2 cl1 cell monolayer after coculture (223). These M cell-like models have been used to investigate the transport of M cell particles (225, 228–232) and the interaction with and entry into M cells of enteroinvasive pathogens such as *Listeria monocytogenes*, *Yersinia enterocolitica*, *Salmonella* spp., and enterovirulent *Escherichia coli* (221). It is noteworthy that a mouse model of M-like cells has been established by El Bahi et al. (226) from cultured mouse intestinal mIC_{Cl2} cells (233) cocultured with lymphocytes freshly isolated from BALB/c mouse PP. When grown on permeable filters, the m-IC_{cl2} cells form sealed, confluent monolayers of cuboid, polarized cells with TJs, develop dense, short apical microvilli, form fluid-transporting domes, conserve the main features of small intestine crypt cells (including the expression of cytokeratins, villin, APN, DPP IV, and glucoamylase), and retain crypt cell features (including intracellular sucrase isomaltase, the accumulation of alpha-L-fucose glycoconjugates, and expression of the polymeric immunoglobulin receptor and the CFTR) (233). m-IC_{cl2} cells have been used to identify the intracellular Toll-like receptor 4 (TLR4) (which recognizes lipopolysaccharide [LPS]) (234, 235), the migration of $CD8^+$ intraepithelial lymphocytes isolated from CCR5-deficient mice infected with *Toxoplasma gondii* (236), and the receptor-mediated intestinal transcytosis of botulinum neurotoxin A (237, 238).

The enteric nervous system, i.e., enteric neurons and enteric glial cells, is a potent modulator of intestinal epithelial barrier function, which has given rise to the novel concept of a digestive "neuronal-glial-epithelial unit" (30). Two coculture models consisting of human submucosa have been described, which contain the submucosal neuronal network cocultured with human fully differentiated mucus-secreting HT29-Cl.16E or enterocyte-like Caco-2 monolayers. These models have been used to investigate the effects of submucosal neuron activation by electrical field stimulation on cell proliferation (239), on the VIPergic neuronal pathway controlling the paracellular permeability and structural organization of TJs (70), and on the protein kinase A (PKA)-independent and mitogen-activated protein kinase (MAPK)-dependent production of IL-8 (240).

Choice of Cells and Technical Considerations

Some considerations must be taken into account when choosing between human colon cancer fully differentiated Caco-2 cells, HT-29 subpopulations, or HT-29 or Caco-2 clone cells as intestinal models for the study of the mechanisms of pathogenesis of enterovirulent bacteria. It is clear that the different human colon cancer cell lines, subpopulations, or clones are not equivalent. For example, the growth rate (94), TER values (93), glucose consumption (94), expression of SI (94) and hexose transporters (123) at the brush border, expression of NHE antiporters at the brush border or basal domain (129, 241, 242), and sucrose activity values (94) all differ considerably in fully differentiated Caco-2 clone cells (243). Analysis of the literature shows that the enterocyte-like models most often used to investigate the molecular and cellular mechanisms of microbial pathogenesis are parental Caco-2 cells (82), Caco-2BBe 1 (86), cl1 (88), and Caco-/TC7 (94) clones, and the HT-29 Rev $Glc^{-/+}$ subpopulation (38). The most frequently used mucus-secreting cell models are the HT29-MTX subpopulation (38) and the HT-29.cl16E clone (42).

Some important technical considerations also have to be taken into account for the appropriate culture of human colon cancer cell lines, subpopulations, or clone cells. Some of the cells that have been transferred between laboratories have been contaminated with mycoplasmas, which affects their functional differentiation. They may, unfortunately, even have been contaminated with human cervical carcinoma epithelial HeLa cells, which makes them unsuitable for use in any studies at all. It is therefore important to obtain cell lines, subpopulations, or clone cells from culture collections or from expert laboratories that can validate that the transmitted cells comply with specifications. The most complete cell polarization and fully functional differentiation have been obtained by culturing the cells on polycarbonate filters. However, the high cost of this culture system has restricted its use to experiments involving TER and transcellular or paracellular passage measurements. In general, cells are cultured in polystyrene tissue culture plates. The cell density during growth can influence the morphological and physiological properties of the fully differentiated cells (243–245). Indeed, some reports describe using protocols in which the culture plates are inoculated with cells at high density after trypsinization. This shortens the culture time as the cells reach confluence quickly, but this is achieved at the cost of good functional differentiation. It is therefore important to carry out regular checks of the polarized organization of the cells by indirect immunofluorescence labeling of SI coupled with a confocal laser scanning microscopy examination and determination of the activity of brush border-associated hydrolases (243, 246). The passage number also influences the morphological and functional differentiation of the cells (243, 247–249). The culture medium used also influences the growth and the functional differentiation of the cells. Indeed, some human colon cancer cells lines, subpopulation, and clones can form multilayers in culture in which only the top layer of cells are fully differentiated (243, 247, 248). This phenomenon has been observed particularly for parental Caco-2 cells when the fetal bovine serum used contains high levels of cyclic AMP (cAMP), and the result is that cell proliferation seems to be preponderant relative to cell differentiation (A. L. Servin, unpublished data). A similar effect has been observed with Caco-2BBe 1 clone cells cultured in culture medium supplemented with glutamine (250, 251). It is therefore necessary to select an appropriate fetal bovine serum that will result in the culture of a cell monolayer composed entirely of structurally and functionally fully differentiated cells (243, 252). These considerations highlight the need to be attentive to the details of the culture conditions described in primary reports.

MECHANISMS OF PATHOGENESIS OF HUMAN ENTEROVIRULENT BACTERIA

Enterovirulent bacteria colonize various sites in the human intestine. *Vibrio cholerae*, *Salmonella enterica* serovar Typhi, enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), and diffusely adherent *E. coli* (DAEC) preferentially affect the small intestine, whereas *Shigella* spp., *Campylobacter* spp., enterohemorrhagic *E. coli* (EHEC), and enteroinvasive *E. coli* (EIEC) infect the colon and *Yersinia* spp., enteroaggregative *E. coli* (EAEC), and *Salmonella* spp. affect both the small and large bowels. By use of adhesive factors and virulence factors, including bacterial effector

proteins, expressed specifically by each pathogen and acting individually or in synergy, enterovirulent bacteria use sophisticated strategies to manipulate the host cell differently (Fig. 2).

A critical step in intestinal bacterial pathogenesis is the ability of enterovirulent bacterial pathogens to interact efficiently with the different cell phenotypes lining the host intestinal barrier. In order to interact with the polarized host epithelial cells that line the intestinal barrier, many bacterial species move within the luminal intestinal compartment by rotating their flagella (253, 254). The molecular mechanism by which the flagellum is assembled involves the regulation of flagellar gene transcription, as well as translational and posttranslational regulation events. For example, the biogenesis of the flagellum in *S. enterica* serovar Typhimurium develops by the coordinated structural assembly of the flagellar proteins that form the flagellar propeller (255–257), and MotA and MotB are cytoplasmic membrane proteins that form the force-generating unit of the flagellar motor (256, 258). The flagella of enterovirulent bacteria have been shown to be necessary for colonizing the intestine, crossing the intestinal mucus layer and then attaching to epithelial cells, and promoting early innate host responses. Human enterovirulent bacteria display huge genetic diversity, and have evolved a wide repertoire of virulence and colonization factors that facilitate host-pathogen interactions with the apical domain of the fully differentiated polarized epithelial cells and M cells that line the intestinal epithelial barrier (259–265). Moreover, human intestinal bacterial pathogens are equipped with a variety of weapons that provide them with a variety of mechanisms for subverting the cellular machinery and circumventing host defenses.

Gram-negative bacteria have developed a variety of solutions for secreting proteins from the cytoplasmic compartment outside the bacterial cell (265–267). Six major protein secretion pathways, numbered I to VI, have been characterized for Gram-negative bacteria (268). The type I secretion system consists of an ATP-binding cassette transporter or a proton antiporter, an adaptor protein that bridges the inner membrane and outer membrane, and an outer membrane pore facilitating the passage of proteins, including cytotoxins belonging to the RTX (repeats-in-toxin) protein family, proteases, lipases, microcins, and colicins, across the cell envelope of Gram-negative bacteria (269, 270). The type II secretion system is a macromolecular, multicomponent structure that translocates a precursor effector protein through the inner membrane by the Sec translocon or the Tat pathway within the periplasm, after which the effector protein is translocated through the outer membrane (271, 272). The type III secretion system (T3SS), composed of more than 20 different proteins which form a large supramolecular structure crossing the bacterial cell envelope, includes the bacterial flagellum and the virulence-associated injectisome, which are two complex, structurally related nanomachines that enterovirulent bacteria use for locomotion and for the translocation of virulence factors into eukaryotic host cells, respectively (273–276). The type IV secretion system is a translocation pore involving the coordinate assembly of core complex proteins, including VirB3 to VirB10, which assembles with VirD4 for substrate recruitment and which, after activation, secretes the substrate (277–279). In addition, by association of the core complex proteins with the VirB11 protein, a pilus formed of VirB2 and VirB5 proteins assembles. The type V secretion system forms a pore in the outer membrane through which the passenger domain passes to the cell surface (280, 281). The newly identified type VI secretion system is a complex bacterial export pathway composed of at least two complexes, a dynamic bacteriophage-like structure and a cell envelope-spanning membrane-associated assembly (282, 283). Like the type III and IV secretion systems, the type VI secretion system translocates substrates directly into recipient cells in a contact-dependent manner.

Enterovirulent noninvasive EPEC and EHEC and enteroinvasive *Yersinia*, *Shigella*, and *Salmonella* use T3SS injectisomes for the infection of host intestinal epithelial cells (Fig. 2). Injectisomes are macromolecular infection machineries consisting of structural and nonstructural proteins which form a basal body anchored in bacterial membranes, an external needle that protrudes from the bacterial surface, and a tip complex that caps the needle, functioning as a bacterial syringe through which bacterial effector proteins are delivered into the cytoplasm of target host cells (284–287). It has recently been revealed that *Shigella* and *Salmonella* injectisome subunit proteins adopt a conserved structure and orientation in their assembled state (288). The analysis of the molecular and cellular mechanisms of virulence of enterovirulent bacteria has shown the great sophistication of the T3SS-associated bacterial effectors. As the result of the piracy of host cell machinery, some enterovirulent bacteria increase the targeting and biochemical activities of their bacterial effectors (289). For example, some bacterial effectors are modified by the attachment of a variety of lipid groups by *S*-palmitoylation or *N*-myristoylation and prenylation, thus facilitating their interaction with the host cell membrane or with membrane lipid raft microdomains. Others modifications allow the optimal targeting of bacterial effectors within the host cell mitochondria or nucleus (289). Following delivery into the host cytoplasm, the bacterial effectors of *Shigella* (290–293), *Salmonella* (291, 293–298), and *Listeria* (293, 299–301) initiate and maintain infection by manipulating host cell biology, such as cytoskeletal dynamics, cell signaling, membrane trafficking, protein ubiquitylation, transcription, and cell cycle progression, and by reprogramming of host cells and circumvention of host defense mechanisms. For EPEC and EHEC pathogenesis, the T3SS injectisome produces an attaching and effacing (A/E) lesion at the microvilli of the brush border. These bacteria bind intimately at the brush border membrane of fully differentiated intestinal epithelial cells, which leads to a localized effacement of absorptive microvilli and the accumulation of host cytoskeletal proteins just beneath the attached bacteria, thus forming the "pedestal" structure of the cell membrane. The capacity for A/E lesion formation is encoded mainly on the locus of enterocyte effacement (LEE) pathogenicity island, the core of which harbors the genes for the T3SS regulators, chaperones, and effector proteins that subvert the host cell cytoskeletal and signaling machinery (302–305). On the other hand, some enterovirulent bacteria produce toxins that, after endocytosis, target the host epithelial cell actin cytoskeleton or microtubule network, impairing the polarized cell sorting of functional proteins, affecting the polarized cell organization, or altering the junctional domain organization (287, 304, 306). Other toxins produce their cytotoxic activity by inducing generally apoptotic death (307). Moreover, enterotoxigenic bacteria deliver cytotonic toxins that after receptor-binding- and endocytosis-activated cell signaling pathways regulating the functionality of membrane-associated proteins supporting specific intestinal functions of absorption/secretion (308, 309) (Fig. 2). Investigation of the pathogenic molecular and cellular mechanisms of enterovirulent bacteria using cultured, fully differ-

entiated human colon cancer cell lines, subpopulations, and clone cells has provided detailed models of their molecular manipulation of host cell biology.

Cell Interaction, Cell Entry, and Intracellular Lifestyle

Enterovirulent bacteria interact with intestinal epithelial cells that form the intestinal barrier via adhesive molecules expressed on their surfaces (Table 2). The biogenesis and regulation of bacterial adhesins in both intestinal and extraintestinal bacterial pathogens have been studied in detail (259–265). Attachment to host intestinal cells is a way of avoiding being dislodged by mucosal secretions and peristalsis, and enterovirulent bacteria produce a wide variety of adhesive structures or factors, including, for example, nonfimbrial and fimbrial polymeric structures that extend out from the bacterial surface and allow them to interact at a distance from the cells. These adhesive factors allow a close association to develop between enterovirulent bacteria and host cell membrane-bound molecules expressing an extracellular domain. The bacterial adhesion to the brush border of enterocytes is much more than just simple attachment. In the case of ETEC, it allows the optimal delivery of cytotonic toxins in the vicinity of their membrane-associated receptors, which is followed by signaling events that affect electrolytes and fluid secretion. Close adhesion allows both EPEC and EHEC to insert the translocated intimin receptor into the host cell membrane, triggering the recruitment of actin immediately underneath the attached bacterium to form the pedestal structures that create and maintain the intimate attachment of the bacterium, resulting in characteristic A/E lesions on the brush border and in dramatically impaired absorption/secretion functions. For *Shigella* and *Salmonella*, adhesion initiates an orderly series of bacterial effector-controlled molecular events within a defined area on the host cell membrane, which facilitate the formation of the dramatic actin-rich cell surface ruffles that are pivotal to the successful completion of bacterial invasion followed by the adoption of an intracellular lifestyle by the internalized bacteria.

Yersinia. The three *Yersinia* species that are human pathogens are *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis*. *Y. enterocolitica* and *Y. pseudotuberculosis* are Gram-negative, food- or waterborne enteropathogenic bacteria that share the same modes of transmission and typically cause self-limiting infections restricted to the intestinal tract and intestinal lymphoid system (310, 311). The core of the *Yersinia* pathogenicity arsenal is the Yop virulon, also known as the "Yop secretion system," expressed by *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*, which allows *Yersinia* to inject specialized proteins, known as Yop effectors, through the plasma membrane into the cell cytoplasm (285, 312).

The cell association and cell entry of *Yersinia pseudotuberculosis* expressing *inv* and/or *ail* loci as a function of intestinal cell differentiation has been investigated by Coconnier et al. (313) using parental undifferentiated and fully differentiated Caco-2 cells (Table 2). This phenomenon parallels the change in the cell distribution of $\alpha_5\beta_1$ integrin, since integrin expression is high at the cell surface of proliferative undifferentiated cells, and the integrin is redistributed so as to localize at the cell-to-cell contacts when the cell reach confluence. Cell invasion occurs when the Caco-2 cells are undifferentiated and is arrested when differentiation commences. Similar differentiation-dependent internalization has been observed for *Y. enterocolitica* in T84 cells (314). In contrast, it has been reported that *Y. enterocolitica* O:8 WA interacts with and is internalized into fully differentiated Caco-2 cells (315, 316). Moreover, the entry of *Y. pseudotuberculosis* and *Y. enterocolitica* into nonphagocytic cells is mediated by the bacterial outer membrane protein (OMP) invasin, and the invasin-mediated uptake requires high-affinity binding of invasin to host cell membrane-associated multiple β1 chain integrin receptors (317, 318).

Shigella. *Shigella* spp. are Gram-negative, enteroinvasive bacteria belonging to the *Enterobacteriaceae* family (290, 292, 319). The genus *Shigella* is divided into four species, *S. boydii*, *S. dysenteriae*, *S. flexneri*, and *S. sonnei*. The World Health Organization (WHO) considers that shigellosis is a significant public health burden in developing countries, with about 160 million cases occurring annually, predominantly in children under 5 years of age, possibly leading to one million deaths per year worldwide. Members of the genus *Shigella* cause bacillary dysentery in humans by invading the colonic epithelial mucosa, inducing a strong inflammatory response, and causing devastating diarrhea. After crossing the epithelium barrier and entering the M cells, *Shigella* bacteria are delivered to resident macrophages, within which they induce apoptosis, and thus reach the basal membrane of fully differentiated intestinal epithelial cells, through which they enter the cells (291). To enter a cell, *Shigella* bacteria use their T3SS needle, which is inserted into the cell membrane to allow the translocation of bacterial effector proteins into the host cell cytoplasm. Once inside the cell, the bacteria escape from the *Shigella*-containing vacuole into the cell cytoplasm, in which the bacteria move through an actin-based motility and spread to the neighboring cells via cellular membrane invaginations known as transpodia (293).

Mounier et al. (320), using fully differentiated Caco-2 cells, were the first to demonstrate that the invasion of human intestinal cells by *S. flexneri* occurs via the basolateral pole of these cells, whereas other bacteria interact with the apical surface without causing any detectable change in the microvilli (Table 2). After bile salt exposure, *S. flexneri* increases the expression of OMP-associated OspE1/OspE2 proteins and displays an increased capacity to adhere to the brush border of fully differentiated T84 cells (321). In fully differentiated Caco-2 cells, Ipa proteins are released from bacteria interacting with the basolateral surface rather than with the apical surface (322). Mutation of the genes encoding the T3SS effectors IpgB1, acting as a functional Rac mimic, and IpgB2, acting as a molecular mimic of Rho for the *Shigella*-induced formation of cell surface lamelliopodia, shows that the cell entry of *S. flexneri* mutants into fully differentiated Caco-2 cells is 70% lower than that of the wild-type strain (323). The *S. flexneri* O antigen, regulated by the WzzB protein, and a very long O antigen have been found to be involved in basolateral cell entry into fully differentiated Caco-2 cells (324). A role of cellular gap junctions in *S. flexneri* pathogenesis has been recently demonstrated. Gap junctions consist of arrays of intercellular channels composed of integral membrane proteins called connexin (Cx) (325). The type of Cx expressed in intestinal cells is still a matter of debate, and little is known about the role of hemichannels in intestinal epithelial cells. Cx26, Cx32, and Cx43 were present in fully differentiated T84 and Caco-2 cells (326–329). Functional data suggest that Cx26-mediated gap junctional intercellular communication plays a crucial role in enhancing the barrier function of TJs in fully differentiated Caco-2 cell monolayers (326). In fully differentiated Caco-2/TC7 cells, Ca^{2+} movements through opening of Cx hemichannels were detected upon *S. flex-*

TABLE 2 Overview of cell interaction, cell entry, and intracellular fate within fully differentiated human colon cancer cell lines

Pathogen	Cell line	Pathogenicity island, virulence factor, or cellular protein	Effect	Reference(s)
Yersinia	Caco-2, T84	Inv	Cell entry blocked by polarization during cell differentiation	313, 314
	M-like Caco-2	Undetermined	Entry within M-like cells	227, 824
Shigella	Caco-2	Undetermined	Specific basolateral cell entry	320
	Caco-2	Ipa	Release of Ipa proteins by bacteria interacting with the basolateral surface	322
	Caco-2	IpgB1, IpgB2	Role in basolateral cell entry	323
	Caco-2/TC7	Connexins	Role of Cx26, Cx32, and Cx43 in basolateral cell entry	328
Listeria	Caco-2	Undetermined	Decrease of cell entry as a function of the polarization during cell differentiation	337, 338, 339
	Caco-2	ActA	Role in cell entry within epithelial cells	340
	C2BBe 1	InlC, Tuba	Role in bacterial spreading	341
Campylobacter	Caco-2	Undetermined	Cell entry	346
	Caco-2	Undetermined	Paracellular translocation	347
	Caco-2	FlaA, FlaB, Mot	Role of flagella and swimming motility for cell entry	350, 351
	T84	Undetermined	PI3K-dependent cell entry	658
	Caco-2	Undetermined	Residing within CD63-positive vacuoles	356
	Caco-2	LuxS	Role in cell entry	364
	T84	FlaA, CadF, PldA, CiaB, CdtA, CdtB, CdtC	Roles in cell entry	352
	T84	CapA	Role in adhesion and cell entry	354
	Caco-2	LOS	Role in cell entry	348
		HtrA, E-cadherin	Role in cell entry	357
Salmonella	Caco-2, T84	Undetermined	Interaction with apical microvilli and cell entry	374–377, 389
	Caco-2	Flagella	Role in association with brush border and cell entry	382–384
	Caco-2	SopA, SopB, SopD, SopE2	Role in cell entry	391
	Caco-2	Undetermined	Internalized bacteria residing within membrane-bound vacuoles	374, 377, 393
	T84	SodC1, SodC2	Role in intracellular survival	394
	Caco-2	MgtBC, PstACS, Iro, SPI-2	Upregulation of genes for magnesium, phosphate, and iron uptake and SPI-2	396
Vibrio cholerae	Caco-2	Undetermined	Adhesion to microvilli	408
	Caco-2	OmpU	Role in cell association	410
	Caco-2	Type IV toxin-coregulated pilus	Role in cell association	411
ETEC	Caco-2	CFAs	Adhesion to microvilli	423
	Caco-2	CSs	Adhesion to microvilli	422, 426–429, 431, 432
	Caco-2	PCFs	Adhesion to microvilli	422, 424
	Caco-2	Antigens	Adhesion to microvilli	423, 425
	Caco-2	Type IV long pilus	Adhesion to microvilli	433
	Caco-2	EtpA and EtpB	Adhesion to microvilli	434
	Caco-2, HT-29 Glc^-	CFAs	Cell differentiation-dependent adhesion of ETEC to microvilli	435, 436
AIEC	Caco-2	Undetermined	Adhesion to microvilli and role of CEACAM6 as cell membrane receptor	415, 456
	Caco-2, T84	FimH	Identification of FimH mutations affecting adhesion	457, 458
	Caco-2BB2, T84	Flagella	Role in cell association and cell entry	449
	T84	OmpC	Role in cell association and cell entry	459
	Caco-2	OMVs	Role of chaperone Gp96 in cell recognition of OMVs	460

(Continued on following page)

TABLE 2 (Continued)

Pathogen	Cell line	Pathogenicity island, virulence factor, or cellular protein	Effect	Reference(s)
tEPEC	Caco-2	Undetermined	Formation of typical tEPEC microcolonies	464
	HT-29 $Glc^{-/+}$, Caco-2, T84	Undetermined	Cell differentiation-dependent formation of typical tEPEC microcolonies	467
	Caco-2	Bundle-forming pili	Role in formation of typical tEPEC microcolonies	465, 466
	Caco-2	Undetermined	Recruitment of cell surface nucleolin around the bacteria present in microcolonies	609
aEPEC	Caco-2	Undetermined	Localized adherence-like, DA and AA patterns of adhesion	487, 488
EHEC	Caco-2, T84	Undetermined	Dense and localized microcolonies at the brush border	464, 492
	Caco-2	LEE	Role in adhesion	493–496
	Caco-2	OmpA, long polar fimbriae	Role in adhesion	497, 498
	Caco-2	Tir	Role in adhesion	496
	Caco-2	YhiE, YhiF, ToxB	Regulators for T3SS-dependent adhesion	499, 500
	Caco-2	Type 4 pili	Role in adhesion	501
	T84	Hemorrhagic *E. coli* pili	Role in adhesion and biofilm formation	502, 503
EAEC	Caco-2, T84	EspP, EhxD	Role in adhesion to brush border	507, 644, 645
	T84	AAF/I and AggR	Role in adhesion to brush border	508
	Caco-2, T84	Undetermined	Cell invasion	509
$Afa/Dr_{DAF/CEACAMs}$ DAEC	Caco-2	Afa-I, Dr, F1845	Role in cell differentiation-dependent diffuse adhesion at the brush border	533–535
	Caco-2/TC7	Dr, F1845	Role in hDAF and hCEA receptor clustering around bacteria adhering onto the brush border	526
	Caco-2/TC7	Dr, F1845, Afa-III	Increased lipid raft-dependent internalization after basolateral infection compared to apical infection	536, 537
			Role in recruitment of β1-integrin around adhering bacteria and preceding cell entry	538, 539
aDAEC	Caco-2	CF16K, CS31A	Role in adhesion at the brush border	432, 517

neri invasion (328). Moreover, in partially differentiated Caco-2/TC7 cells, *S. flexneri* is captured at a distance from the cell surface on the apical side of the cell-to-cell domain by nanometer-thin micropodial extensions (330). This phenomenon is accompanied by F-actin condensation and cell entry via an extracellular signal-regulated kinase 1/2 (Erk1/2) MAPK-dependent process involving the T3SS tip complex proteins and stimulated by ATP- and Cx-mediated signaling.

Movement of *Shigella* within the host cell cytoplasm requires F-actin polymerization, which allows *Shigella* to induce F-actin nucleation and elongation, thus gaining propulsive force. This bacterial movement causes the cell membranes of primarily infected polarized cells to protrude, forming transpodia allowing the penetration of the intracellular bacteria into neighboring cells and thereby allowing the bacteria to disseminate into adjacent cells. It is noteworthy that the roles of the *S. flexneri* virulence factors involved in intercellular spread of *S. flexneri* or cell-to-cell passage via transpodia have been investigated using plaque assay consisting of nonintestinal undifferentiated epithelial cells such as HeLa cells (331) or isolated Caco-2 cells or islets of undifferentiated Caco-2 cells (332, 333).

Listeria. *Listeria monocytogenes* is a ubiquitous, Gram-positive bacterium that thrives in diverse environments such as soil, water, various food products, human beings, and animals (300, 301, 334). The disease caused by this bacterium, listeriosis, is acquired by ingesting contaminated food products and mainly affects immunocompromised individuals, pregnant women, and newborn infants. It is responsible for severe systemic infections with high mortality rates, including meningitis or meningoencephalitis, septicemia, diarrhea, miscarriages, and perinatal infections. After being ingested, this intracellular pathogen breaches the intestinal barrier. During severe infections it crosses the blood-brain barrier, resulting in infection of the meninges and the brain, and in pregnant women it crosses the fetoplacental barrier, leading to infection of the fetus. *L. monocytogenes* displays a battery of virulence factors, some of which functionally or structurally mimic host proteins to hijack host cellular processes (299, 301, 335, 336), and several different host cell signaling cascades during its intracellular life cycle (293).

The first report describing the interaction and cell entry of an enterovirulent bacterium with the cultured Caco-2 cells was made by Gaillard et al. (337) (Table 2). These authors used semiconfluent, mainly undifferentiated cell monolayers and reported that *L. monocytogenes* entered the cells and that the internalized bacteria resided inside vacuoles. These authors also observed that listeriolysin O (LLO) functions as a major factor allowing bacteria to escape from phagosomes and to multiply within the cell cytoplasm. Karunasagar et al. (338), using fully differentiated Caco-2 cells, reported that *L. monocytogenes* entered the cells through the apical surface without modifying the microvilli but as the result of

forming lamellipodia that are involved in the cellular uptake of the bacteria. Gaillard and Finlay (339) have investigated the entry of *L. monocytogenes* into Caco-2 cells as a function of cell polarization and differentiation. *L. monocytogenes* entered through the entire surface of undifferentiated cells but predominantly through the basolateral surface of polarized cells, since the numbers of internalized bacteria decreased dramatically when the Caco-2 monolayers were cultured beyond confluence. Interestingly, by creating a disruption of intercellular junctions in Caco-2 monolayers that expose the lateral domain of the fully differentiated cells, an enhanced number of bacteria were internalized after apical infection. Loss of the actin-polymerizing protein ActA reduces the apical entry of *L. monocytogenes* into fully differentiated Caco-2 and MDCK epithelial cells (340). Scanning electron microscopy examination of infected, fully differentiated Caco-2 cells suggests that internalization involves microvilli that mediate bacterial uptake after being remodeled to form pseudopods. In contrast, ActA does not contribute to *L. monocytogenes* internalization by COS-1 fibroblasts or Hepa 1-6 hepatocytes, indicating that ActA can direct an internalization pathway specific to epithelial cells. It has been established in undifferentiated Caco-2 cells that InlA interacts with human E-cadherin to promote *L. monocytogenes* cell entry through a dynamic process involving coordinated actin cytoskeleton rearrangements and host cell membrane remodeling at the site of bacterial attachment. Bacterial and host proteins that directly regulate *L. monocytogenes*-induced protrusions have been identified using fully differentiated Caco-2BBe 1 clone cells, and this showed that the spread of the bacterium between polarized cells requires secreted protein InlC and cell adaptor protein Tuba (341). Indeed, Tuba functions as a ligand of InlC. InlC binds to a carboxy-terminal SH3 domain in Tuba that normally engages the human actin regulatory neural Wiskott-Aldrich syndrome protein (N-WASP). Since Tuba and N-WASP are known to control the structure of apical junctions in epithelial cells, it has been proposed that InlC may promote protrusion formation by inhibiting Tuba and N-WASP activity, probably by impairing the binding of N-WASP to the Tuba SH3 domain. Experiments with myosin II inhibitors indicate that InlC-mediated disruption of apical junctions accounts for the role of this bacterial protein in protrusion formation.

It is noteworthy that as for *Shigella*, isolated undifferentiated Caco-2 cells, islets of undifferentiated Caco-2 cells, or undifferentiated Caco-2 cells at the early stage of confluence have been used to identify *Listeria* virulence factors involved in the intracellular lifestyle, including intracellular movements (342, 343).

Campylobacter jejuni. *Campylobacter* spp. are microaerophilic, curved, Gram-negative rods exhibiting motility and are carried in the intestines of many wild and domestic animals, particularly avian species, including poultry (344). *Campylobacter* is recognized as the leading cause of bacterial food-borne diarrheal disease worldwide. Symptoms can range from mild to serious infections in children and the elderly to permanent neurological symptoms. *Campylobacter* spp. express a set of virulence factors, including flagella for swimming within the mucus and host-bacterium interactions, chemotaxis proteins, adhesive factors (including CadF, which recognizes fibronectin), Peb1, Cj1496c, JlpA (which activates NF-κB and p38), mitogen-activated protein kinase (MAPK), lipoprotein CapA (implicated as a possible adhesin), the secreted protein CiaB (which is required for the invasion of epithelial cells), and cytolethal distending toxin (CDT), (345). *Campylobacter* spp. lack T3SS and invade intestinal epithelial cells, but the mechanisms that control cell entry are not fully understood. It is clear that cell entry involves a microtubule-dependent mechanism, since the pseudopods entrapping cell-associated bacteria contain microtubules. In addition, several *Campylobacter* strains also require microfilament polymerization for cell entry. Once internalized, *C. jejuni* localizes within vacuoles and moves along the microtubules to the perinuclear region of the cell. The role of these internalized bacteria in pathogenesis, possibly by evading the immune system and establishing a protected reservoir, remains to be determined.

Using fully differentiated Caco-2 cell monolayers grown on microporous membrane filters, Konkel et al. (346) were the first to observe that *C. jejuni* translocates across the cell monolayers by passing both through and between cells (Table 2). When infecting a T84 cell monolayer via the basal domain, *C. jejuni* translocation appears to occur via a paracellular route rather than a transcellular route (347). *C. jejuni* isolates expressing ganglioside-like lipooligosaccharide (LOS) are highly adhesive and penetrate into fully differentiated Caco-2 and T84 cells, in contrast to *C. jejuni* isolates that lack these structures (348). Cell association and invasion of Caco-2 cells are inhibited by various sugars, including D-glucose, D-mannose, D-fucose, and *N*-acetylneuraminic acid (349, 350), and intracellular *C. jejuni* is localized within membrane-bound vacuoles (350). Examination of the role of flagella and swimming motility in the interactions of *C. jejuni* with fully differentiated Caco-2 cells reveals that both the flagellated, nonmotile (*flaA*$^+$ *flaB*$^+$ *mot*) and nonflagellated, nonmotile (*flaA flaB mot*) mutants, unlike the wild-type strain, are unable to translocate across cell monolayers (350–352). Moreover, in the presence of intestinal mucus, *C. jejuni* displays straight swimming motility punctuated by tumbling (353). An autotransporter protein *capA* insertion mutant has a significantly lower capacity to associate with and invade fully differentiated Caco-2 cells (354). During *C. jejuni* translocation across fully differentiated Caco-2 cell monolayers, the absence of any change in TER and inulin paracellular passage indicates that the bacteria translocated through the cytoplasm of the cells invade, rather than via intercellular spaces (355). *C. jejuni* invading, via an actin- and microtubule-independent mechanism, fully differentiated Caco-2 cells localized in at the periphery of large islets of cells have been found residing within CD63-positive vacuoles, in which they are metabolically active (356). The serine protease HtrA of *C. jejuni* plays a role in promoting the basolateral invasion of fully differentiated Caco-2 by cleaving E-cadherin (357).

Quorum sensing (QS) is a "language" by which bacteria and host cells communicate (358–360). Signaling factors produced by enterovirulent bacteria have the function to communicate both the cell density and the metabolic potential of the environment and also to regulate the expression their virulence factors (361–363). Consistent with the fact that the genome sequence of *C. jejuni* NCTC 11168 contains a gene encoding an orthologue of LuxS, which is required for QS autoinducer-2 (AI-2), a *luxS* mutant displays the same ability to invade fully differentiated Caco-2 cell monolayers as the parental strain, even though it exhibits decreased motility in semisolid media, suggesting that quorum sensing may play a role in the regulation of motility (364).

When *C. jejuni* is grown in iron-limited medium in the presence of norepinephrine (NE), the motility of bacteria and their entry into fully differentiated Caco-2 cells are both increased

(365). In addition, in the presence of NE, *C. jejuni* causes greater disruption of cultured epithelial cell monolayers than in the absence of NE. The roles of environmental stress factors, including temperature shift, nutrient starvation, and atmospheric oxygen concentration, in *C. jejuni* pathogenicity have been evaluated in fully differentiated Caco-2 cell monolayers (366–368). Nutrient insufficiency and temperature elevation both transiently affect bacterial growth and also affect the adhesion and invasive properties of *C. jejuni*. Oxidative stress does not affect either the binding or invasion of cells, whereas oxygen exposure or microaerobic conditions increase both the invasion capability and survival of intracellular *C. jejuni*.

***Salmonella* spp.** Salmonellae are Gram-negative bacteria that cause gastroenteritis and enteric fever (295). *Salmonella* serovars associated with gastroenteritis orchestrate a strong intestinal inflammatory response and cause deleterious structural and functional cell injuries that result in severe secretory disease. *Salmonella* bacteria share the ability to invade the host by inducing their own uptake, and they survive and multiply within the epithelial cells and M cells lining the intestinal epithelium (369). To do this, *Salmonella* virulence requires the coordinated expression of complex arrays of virulence factors (369, 370). The most important *Salmonella* virulence genes are those located within the five so-called *Salmonella* pathogenicity islands (SPIs) (369, 370). *Salmonella* cells attach to the enterocytes and M cells by means of adhesions, including those encoded within SPI-3 and SPI-4. Invasion of adhering *Salmonella* within the cells occurs by an F-actin-dependent process triggering a cell membrane engulfment mediated by virulence factors encoded within SPI-1 and SPI-5. Both T3SS-1 and T3SS-2 are composed of approximately 20 to 30 proteins, with a major subset of these proteins having a structural role in forming the supramolecular injection apparatus, known as the needle complex, which is composed of three distinct substructures: a multiring base, an inner rod, and a needle (286, 371). Another set of proteins forms the translocon, thus producing a pore in the host cell membrane allowing the delivery of bacterial effectors into the host cell cytoplasm (286, 294, 372). Moreover, both T3SS-1 and T3SS-2 are responsible for delivering a series of specific bacterial effectors into host cells, altering host cell organization, functions, and survival. When internalized, bacteria reside within the cell cytoplasm within large vesicles named *Salmonella*-containing vacuoles (SCVs), where they replicate. For the intracellular lifestyle, virulence factors dependent on SPI-2 and the plasmid pSLT (a cryptic plasmid present in *S.* Typhimurium strain LT2) are essential for survival (298, 373). The SCVs transcytose to the basolateral membrane and release the bacteria to the submucosa, in which they are internalized within resident phagocytes again within SCVs, where SPI-3 in addition to SPI-2 and the pSLT plasmid plays an important role. Lastly, the infected phagocytes can disseminate through the lymph and the bloodstream. Alternatively, bacteria can also be directly taken up by dendritic cells (DCs) from the submucosa (370). In addition, emerging evidence indicates that these effectors are also modular proteins consisting of distinct functional domains/motifs that are utilized by the bacterium to activate intracellular signaling pathways that modify host cell functions (370).

The first reports describing the interaction of *S.* Typhimurium with fully differentiated Caco-2 cells and the cellular consequences were from Finlay's group (374–377) (Table 2). Adhesion of *Salmonella* at the brush border of fully differentiated Caco-2 cells has been observed to involve the SPI4-dependent, very large nonfimbrial adhesin SiiE (378) and Std fimbriae (379). It has been suggested that a Galβ(1-3) GalNAc epitope located in the glycocalyx is involved in the early recognition events between *S.* Typhimurium and Caco-2 BBe clone cells (380). Moreover, SadA, a purported trimeric autotransporter adhesin of *S.* Typhimurium, appears to be involved in cell aggregation and biofilm formation, and it increases adhesion to fully differentiated Caco-2 (381). In addition, it has been observed that flagella of *S. enterica* serovar Enteritidis are involved in the association with and invasion of fully differentiated cells (382–384). Using three of the four classes of mutants that remain virulent in mice, Betts and Finlay (375) observed that *S.* Typhimurium invasiveness requires intact motility and at least six distinct genetic loci. CorA, the primary or "housekeeping" Mg^{2+} channel, appears to be involved in the expression of several *S.* Typhimurium virulence factors, since a *corA* mutant strain loses swimming motility and expresses lower levels of InvH and SipC, accompanied by a decreased ability to invade fully differentiated Caco-2 cells (385). Overexpression of matrix metalloproteinase 9, which regulates the production of MUC-2 mucin, increases *S.* Typhimurium adhesion to fully differentiated, mucin-secreting HT-29 cl.16E cells (386). Adherence of Std-fimbriated *S.* Typhimurium to fully differentiated Caco-2 cells is blocked by H type 2 oligosaccharide (Fucα1-2Galβ1-4GlcNAc) (387). In addition, sialic acid plays a role in the adhesion of *S. enterica* serovar Typhi to fully differentiated Caco-2 cells (388).

Finlay and Falkow (377) reported for the first time that *S.* Typhimurium, after interacting with the well-organized apical microvilli of fully differentiated parental Caco-2 cells, induced a dramatic, localized reorganization of the F-actin cytoskeleton at the site of bacterial attachment known as membrane ruffling (377, 389). Following the disruption of the cell membrane, *S.* Typhimurium penetrated the cells and appeared in the basolateral medium of the fully differentiated Caco-2 cell monolayer. F-actin filament rearrangement and morphological changes at the apical domain of Caco-2 cells are essential for the entry of *S.* Typhimurium (374–377). The T3SS-1 effector SipA, which directly binds F actin, thus modulating actin dynamics and facilitating bacterial entry, has been found to be preferentially associated with peripheral cortical F-actin filaments but not with stress fibers in infected fully differentiated Caco-2 cells (390). The T3SS-1 effectors SopE2 and SopB are required for invasion, whereas the SipA protein accelerates entry into epithelial cells. A contribution of the T3SS-1 effectors SopA and SopD to the invasion of nonpolarized T84 cells by *S.* Typhimurium has been observed, whereas in contrast, SopA, SopB, SopD, and SopE2 all increase the invasiveness of an SipA-positive strain in polarized T84 cells (391). These observations in fully differentiated Caco-2 and T84 cells have been validated in a human intestinal *in vitro* organ culture system in which *S.* Typhimurium has been shown to interact with border-expressing cells and induce membrane ruffles (392).

Internalized *S.* Typhimurium cells are enclosed in SCVs within the cytoplasm (374, 377). It has been noted that *S.* Typhi and *S.* Typhimurium use similar mechanisms to invade and carry out intracellular trafficking in Caco-2 cells (374, 393). The biogenesis of SCVs within mammalian cells has been intensively studied over recent years using various different polarized epithelial cells. The ability of *S. enterica* serovar Choleraesuis to survive within fully differentiated Caco-2 cells has been found to depend on *sodC* genes, with *sodC2* being more important than *sodC1* (394). Inves-

tigation of the role of T3SS-2 in cell entry, survival, and proliferation within the cells has revealed more efficient entry into fully differentiated Caco-2 and T84 cells than into HeLa cells (395). Moreover, although T3SS-dependent intracellular proliferation has been observed in HeLa cells, intracellular replication in fully differentiated cells was severely restricted and was not affected by SPI-2 deletion (395). Analysis of the transcriptome of *S.* Typhimurium in fully differentiated Caco-2 cells has been conducted and compared to *Salmonella* gene expression inside macrophages (396). Upregulation of the *mgtBC*, *pstACS*, and *iro* genes for magnesium, phosphate, and iron uptake and of the SPI-2 pathogenicity island has been observed. Moreover, the invasion-associated SPI-1 pathogenicity island, and the genes involved in flagellar biosynthesis were expressed inside epithelial cells at later stages of the infection, whereas they were constantly downregulated in macrophage-like cells. The ability of *Salmonella* to sense and adapt to the intracellular environment of different types of host cells has also received attention. *S.* Typhimurium replicating in the *Salmonella*-containing vacuole in undifferentiated Caco-2 cells utilized glucose but not glucose 6-phosphate or other phosphorylated carbohydrates, gluconate, or fatty acids as their major carbon substrate (397). Carbohydrate phosphotransferase systems were encoded by the genes *ptsG/crr*, *manXYZ*, *fruBA*, *malX/crr*, *scrA/crr*, and *bglF*, and glucose 6-phosphate was taken up by both pathogens via the UhpT transporter, which is under the control of a complex two-component system (*uhpABC*). *S.* Typhimurium mutants defective for the uptake of glucose and mannose (Δ*ptsG manXYZ*) and glucose 6-phosphate (Δ*uhpT*) have less capability for intracellular replication in undifferentiated Caco-2 cells, and the Δ*ptsG manXYZ uhpT* triple mutant is still able to replicate within the vacuoles (398).

Vibrio cholerae. The facultative pathogen and Gram-negative bacterium *Vibrio cholerae* is the causative agent of cholera, which is responsible for significant mortality and economic damage (399–401). During its life cycle, *V. cholerae* has both a human stage and an environmental stage. *V. cholerae* is differentiated serologically on the basis of the O antigen of its lipopolysaccharide. The O1 serogroup has been subdivided into two phenotypically distinct biotypes: El Tor and classical. Cholera toxin (CT)-producing strains of the O1 and O139 serogroups cause the vast majority of cases of the disease worldwide. The major virulence factors of toxigenic *V. cholerae* are CT, which is encoded by a lysogenic filamentous bacteriophage (CTXPhi), and toxin-coregulated pilus (TCP), an essential colonization factor that is also the receptor for CTXPhi (402, 403). The type IV TCP acts as a receptor for CTXPhi, contributes to the secretion of the colonization factor TcpF, and contributes to microcolony formation by mediating bacterium-bacterium interactions. In addition to CT, *V. cholerae* produces other putative toxins, such as the zonula occludens toxin (Zot) (404) and accessory cholera enterotoxin (Ace) (405). The role of Zot in *V. cholerae* pathogenesis is a subject of debate (403). Rearrangements occurring in the water environment in virulent *V. cholerae* strains have been proposed as one of the mechanisms of formation of clones with an incomplete or no prophage. Interestingly, it has been shown that variability of the CTXPhi prophage genome is an important factor in the modification of *V. cholerae* virulence potential, determining the severity of the infection (406, 407).

Panigrahi et al. (408) were the first to report the adhesion of non-O1 *V. cholerae* strain NRT36S to the microvilli of fully differentiated Caco-2 cells (Table 2). Adhesion of *V. cholerae* has also been investigated using fully differentiated mucus-secreting HT-29-18 N2 cells (409). *V. cholerae* uses its OmpU outer membrane protein to adhere to the brush border of fully differentiated Caco-2 cells (410). The type IV TCP of *V. cholerae* has three functions: it acts as a receptor for CTXPhi, the lysogenic filamentous bacteriophage that carries the CT genes in epidemic *V. cholerae* strains; it secretes the colonization factor TcpF and contributes to microcolony formation by mediating bacterium-bacterium interactions; and it acts as an attachment factor for binding to fully differentiated Caco-2 cells, since attachment was defective in mutants lacking TCP compared to in the wild type (411).

Enterovirulent *E. coli*. Six classes of human enterovirulent *E. coli* have been defined: ETEC, EPEC, EHEC, EAEC, EIEC, and DAEC (304, 412–414). In addition, the existence of a particular group of *E. coli* strains named adherent-invasive *E. coli* (AIEC) has recently emerged from studies investigating mucosa-associated bacteria in patients with inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD) (415–418). ETEC, EPEC, EHEC, and EAEC interacted with the brush border of mature enterocytes and remained extracellular. Nataro et al. (419), examining adhesion of diarrheagenic *E. coli* to cultured nonintestinal undifferentiated epithelial Hep-2 cells, first observed three different patterns of adhesion: diffuse adherence (DA), in which bacteria cover the whole surface of the cell, localized adherence (LA), in which attachment is limited to one or a few sites on the cell surface, and aggregative adherence (AA), in which adhering bacteria aggregate in an unorganized fashion. Only EIEC and AIEC are capable of entering and replicating within enterocytes.

(i) ETEC. Enterotoxigenic *E. coli* strains are a main cause of diarrhea in young children under 5 years of age in developing countries and also in adult travelers visiting areas of endemicity (420). ETEC produces a cholera-like, watery diarrheal disease by adherence, involving adhesive factors, onto brush border-associated receptors in mature enterocytes and expression and delivery of heat-labile enterotoxin (LT) and/or heat-stable enterotoxin (ST)-recognizing, brush border-associated receptors activating cAMP or cGMP, respectively, and cell signaling controlling intestinal transports (308, 412, 421). ETEC attaches to microvilli via filamentous bacterial surface structures, known as colonization factors (CFs), and more than 20 different CFs have been described. A family of ETEC adhesive factors that includes colonization factor antigens (CFAs), *E. coli* surface antigens (CSAs), and PCFO71 has been classified with the class 5 fimbriae on the basis of the complete DNA sequences of the gene clusters encoding CFA/I, CS1, CS2, the major fimbrial subunit, and outer membrane protein (OMP) and of the primary sequence of the major fimbrial subunit, CFA/I, and related fimbriae, (422).

Darfeuille-Michaud et al. (423) were the first to have reported that ETEC strains expressing colonization factor antigen I (CFA/I), CFA/II, CFA/III, and antigen 2230 adhere to isolated human enterocytes (Fig. 8A and Table 2). Other ETEC adhesive factors conferring adhesion to fully differentiated Caco-2 cells have been identified, including adhesive factor PCFO20 (424), antigens 8786 (425), CS4 (422), CS6 (426), CS14 (422), CS17 (422), CS19 (427), CS20 (428), CS22 (429, 430), CS23 (431), and CS31A (432), PCF071 (422), type IV long pilus encoded by *ingA* (433), high-molecular-weight glycosylated protein EtpA (434), and the putative EtpB transporter (434). Adhesion of ETEC strains develops at

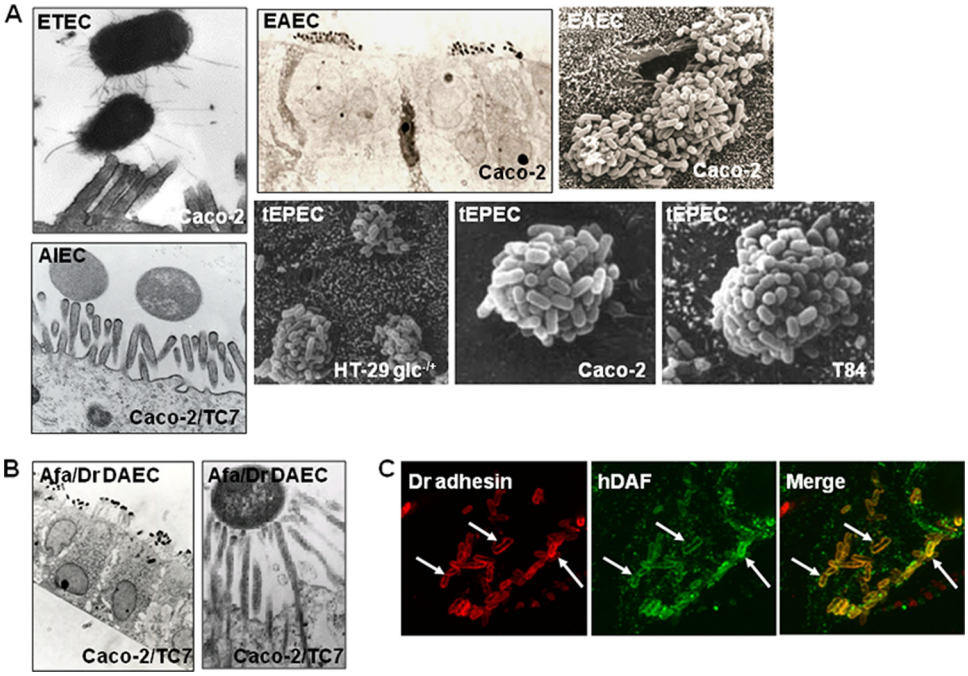

FIG 8 Observation of enterovirulent bacteria adhering to the brush border of cultured human fully differentiated colon cancer cells. (A) Scanning and transmission electron microscopy micrographs show interaction of CFA/I-positive ETEC with microvilli of fully differentiated parental Caco-2 cells (immunolabeling reveals the ETEC fimbrial adhesins mediating attachment), EAEC bacteria forming clusters of bacteria at the brush border of fully differentiated parental Caco-2 cells, interaction of AIEC with microvilli of fully differentiated Caco-2/TC7 clone cells, and typical, well-formed and compact tEPEC microcolonies at the brush borders of fully differentiated HT-29 $Glc^{-/+}$, parental Caco-2, and T84 cells. (B) Transmission electron microscopy micrographs show the diffuse adhesion of Afa/Dr $DAEC_{DAF/CEACAM}$ on the brush border of fully differentiated Caco-2/TC7 clone cells. Note that a single bacterium attracted numerous microvilli. (C) Confocal laser scanning microscopy examination of immunofluorescence labeling micrographs shows the receptor clustering around Afa/Dr $DAEC_{DAF/CEACAM}$ bacteria adhering onto the brush border of fully differentiated Caco-2/TC7 clone cells. Double-immunofluorescence labeling is with anti-DAF antibody (green) and anti-Dr adhesin antibody (red). (The three tEPEC micrographs in panel A are reprinted from reference 467 with permission from the International Society of Differentiation; the micrographs in panel B are reproduced from reference 875 with permission from BMJ Publishing Group Ltd.)

the brush border without modifying the structure of the microvillus (423). The expression of ETEC CFA receptors at the brush borders of fully differentiated, HT-29 $Glc^{-/+}$, and Caco-2 cells develops as a function of cell differentiation and appears to be controlled by the presence of glucose during cell culture (435, 436). ETEC bearing the fimbrial colonization factor antigens CFA/I, CFA/II, and CFA/III and the nonfimbrial antigen 2230 interacted with the mucus produced at the apical domain of fully differentiated mucin-secreting HT29-MTX cells (437). It was noted that ETEC strains have been found to be invasive in the undifferentiated ileocecal adenocarcinoma HCT-8 cells (216).

(ii) EIEC. Enteroinvasive *E. coli* strains can cause diarrhea (304, 413, 414). EIEC strains are biochemically, genetically, and pathogenically closely related to *Shigella* spp. Like *Shigella*, EIEC infects the colonic mucosa. The pathogenesis of EIEC has not yet been fully elucidated. EIEC can be distinguished from *Shigella* by a few minor biochemical tests, but these pathotypes share several identical virulence factors (412–414, 438, 439). The genes necessary for EIEC cell entry are carried on a 140-MDa plasmid designated pInv. Prominent among these genes are the *mxi* and *spa* loci, which encode a T3SS that allows the secretion of numerous proteins, including the effectors of the invasion phenotype Ipa proteins such as IpaA, IpaB, IpaC, and IpaD. The escape of internalized EIEC from the endocytic vacuole into the cytoplasm is followed by an actin-dependent motility movement, which leads to the passage of the bacteria into neighboring cells.

EIEC strains growing in the cytosol of undifferentiated confluent Caco-2 cells utilize glucose, but not glucose 6-phosphate, or other phosphorylated carbohydrates, gluconate, or fatty acids as their main carbon substrates (397, 398) (Table 2). Moreno et al. (440) have compared the intracellular lifestyle of EIEC within fully differentiated Caco-2 cells at the cellular and molecular levels to that of *S. flexneri*. In a plaque assay of confluent undifferentiated Caco-2 cells, the levels found for EIEC and *S. flexneri* were similar at 1, 2, and 3 h postinfection, but at 4 and 5 h postinfection, fewer intracellular EIEC cells were found, suggesting lower intracellular proliferation than for *S. flexneri*. EIEC displays significantly less-efficient cell-to-cell passage in the plaque assay than *S. flexneri*, because the plaques formed by EIEC are four times smaller than those formed by *S. flexneri*. Examined at the molecular level, expression of the regulatory gene *virB* and the invasion virulence genes *ipaA*, *ipaB*, *ipaC*, and *ipaD* by EIEC during invasion within Caco-2 cells is lower than that during *S. flexneri* infection. Expression of the regulatory genes *virB* and *virF*, the invasion virulence genes *ipaA*, *ipaB*, *ipaC*, and *ipaD*, and *iscA*, which is necessary for actin tail assembly by EIEC during cell-to-cell spreading, is lower than that observed during *S. flexneri* infection. In contrast, the regulatory gene *virF* is the only gene to be expressed to a greater extent by EIEC than by *S. flexneri* (440).

(iii) AIEC. A heterogeneous group of *E. coli* strains have been recently isolated from mucosa-associated bacteria in patients with IBD, including UC and CD (415–418). It has been proposed that

these *E. coli* strains may constitute a new, potentially pathogenic *E. coli* group designated adherent-invasive *E. coli* (AIEC) (205), which display (i) the ability to adhere to cultured human intestinal cells with an adhesion index equal to or greater than 1 bacterium per cell, (ii) the ability to invade cultured human intestinal cells with an invasion index equal to or greater than 0.1% that of the original inoculums (205), (iii) the involvement of host cell actin polymerization and microtubule recruitment in bacterial uptake, (iv) the ability to survive and to replicate within macrophages, and (v) the absence of any known cell invasion determinants. It has been hypothesized that the LF82 genome has evolved from those of extraintestinal pathogenic *E. coli* (ExPEC) B2 strains by the acquisition of *Salmonella* and *Yersinia* isolated or clustered genes or predicted coding sequences located on plasmids and at various loci on the chromosome (441). Following the isolation of the prototype AIEC LF-82 strain, other CD- and UC-associated AIEC strains have been isolated and characterized (442–449). The results indicate that AIEC isolated from IBD patients has not evolved from a single ancestral background but corresponds to a group of bacteria that have been able to take advantage of an "IBD microenvironment" and probably share some common genes with ExPEC (446). Effectively, ExPEC and AIEC strains share similar virulence gene sets, and certain strains are phylogenetically related to the B2 phylogroup, but most ExPEC strains do not behave like AIEC strains (450–452). Analysis of the genomes of *E. coli* isolates obtained from patients with UC and CD (453) and of the genomes of AIEC strains LF82 (441), UM146 (452), NRG857c (O83:H1) (451), and HM605 (450) reveals that the genome of AIEC is characterized by the absence of known virulence factors composing the T3SS of enteroinvasive *Salmonella*, *Yersinia*, and *Shigella* strains and by the presence of a number of ExPEC-related virulence determinants such as the *pap*, *sfa*, *cdt*, *sat*, and *hly* genes and of the genes of ExPEC-associated genomic islands, but importantly, with little or no evidence of group-specific determinants. However, it is impossible to exclude the possibility that AIEC expresses so-far-unidentified AIEC-specific genes that could be IBD specific (451). On the basis of what is known about AIEC pathogenesis and the absence of any demonstration that AIEC is directly involved in the development of IBD lesions, so far it seems reasonable to think that AIEC strains constitute a heterogeneous new pathovar of enterovirulent *E. coli* with genomic profiles that are indistinguishable from those of ExPEC isolates but which display particular proinflammatory virulence traits.

AIEC strains isolated from ileal specimens from CD patients with chronic lesions and early recurrent lesions, including strain LF82, have the ability to adhere onto the brush borders of fully differentiated Caco-2 (205, 415, 416, 418) and T84 (454, 455) cells (Fig. 8A and Table 2). The role of human CEACAM6 has been demonstrated *in vitro* and *in vivo*. In isolated human ileal enterocytes, LF82 adhesion at the brush border was inhibited by an anti-CEACAM6 antibody (456). Moreover, in fully differentiated Caco-2 cells, colocalization between CEACAM6 and adherent LF82 bacteria has been observed at the brush border (456). AIEC strains LF9. LF15, LF31, and LF82 entered within fully differentiated T84 cells at a level 2.5-fold lower than *S.* Typhimurium (454). When examining mucosa-associated FimH-positive *E. coli* strains isolated from IBD and non-IBD pediatric patients for their ability to bind onto fully differentiated Caco-2 cells, the results showed a higher site substitution rate in the *fimH* gene, which, together with a higher number of mutations, influences the adhesiveness of the strains (457). Examining the *fimH* genes of AIEC and non-AIEC strains, Dreux et al. (458) concluded that AIEC strains predominantly express FimH with amino acid mutations of a recent evolutionary origin. Moreover, the authors showed that point mutations in FimH confer on AIEC bacteria a significantly higher ability to adhere to CEACAM-expressing fully differentiated T84 cells. Interestingly, the replacement of *fimH* from LF82 with *fimH* from *E. coli* K-12 decreases the ability of bacteria to persist and to induce severe colitis and gut inflammation in infected CEABAC10 transgenic mice. The role of OmpC in the adhesion and cell entry of LF82 has been demonstrated using fully differentiated T84 cells (459). AIEC strains LF82 and O83:H1 display a similar ability to adhere to and invade fully differentiated Caco-2BBe 2 clone cells and T84 cells (449). In AIEC O83:H1, the flagellum plays a pivotal role in the adhesion to and invasion of fully differentiated Caco-2BBe 2 clone cells and T84 cells, since a nonflagellated *E. coli* O83:H1 strain loses both adhesiveness and invasiveness (449). Several bacterial pathogens produced outer membrane vesicles (OMVs) for delivery of virulence factors into host intestinal cells. In fully differentiated Caco-2 cells, LF82 infection promotes the apical overexpression of endoplasmic reticulum (ER)-localized stress response chaperone Gp96, which acts as a receptor for LF82 OMVs and in turn plays a role in the internalization of LF82 bacteria (460).

(iv) EPEC. EPEC strains are a frequent cause of infantile diarrhea in the developing world (302, 304, 413, 414). EPEC strains typically produce LA by forming randomly distributed well-formed and compact microcolonies at the cell surface of intestinal cells expressing a brush border (461). Recently, two subclasses of EPEC have been defined and designated typical EPEC (tEPEC) and atypical EPEC (aEPEC) (462, 463).

Knutton et al. (464) were the first to report the presence of well-formed and dense tEPEC microcolonies in fully differentiated Caco-2 cells (Fig. 8A and Table 2). LA of tEPEC is mediated by the bundle-forming pili (BFP), a type IV pilus encoded by pEAF which interconnects bacteria within the dense microcolonies, thus promoting their particular adhesion organization in the brush borders of fully differentiated Caco-2 cells (465, 466). tEPEC microcolonies have been also observed on infected fully differentiated HT-29 $Glc^{-/+}$ and T84 cells, and the formation of tEPEC microcolonies increases as the brush border develops during the cell differentiation of Caco-2 cells (467). Importantly, animal EPEC-like pathogens such as *Citrobacter rodentium* (468), rabbit diarrheagenic *E. coli* (RDEC-1) (469–477), and rabbit EPEC (REPEC) (472, 476–484), which express virulence factors similar those of human tEPEC, have been shown to produce identical structural and functional damage in animal intestinal barriers and in cultured, fully differentiated human intestinal cells.

It has been shown that the basic difference between tEPEC and aEPEC is the presence of pEAF in tEPEC and its absence in aEPEC (485, 486). Localized adherence-like (LAL), DA, and AA patterns have all been observed for aEPEC strains adhering to fully differentiated Caco-2 cells (487, 488).

(v) EHEC. The noninvasive EHEC strains are the cause of hemorrhagic colitis, nonbloody diarrhea, acute intestinal inflammation, and hemolytic uremic syndrome (304, 413, 414, 489). EHEC strains have evolved from EPEC strains by acquiring bacteriophages that encode Shiga-like toxins (Stx) (307), but there are clear differences between EPEC and EHEC pathogeneses (305, 490). Stx-producing EHEC, mainly the O157 strains, have been

classified as locus of enterocyte effacement (LEE) positive in the pathogenic group of Stx-producing *E. coli* (STEC) (491). It has been noted that some other strains of STEC are LEE negative, including serogroups O26, O45, O103, O111, and O145 (491). Strains of EHEC belonging to serogroup O157 are most commonly associated with severe human diseases and express specific sets of virulence genes, including those encoding Shiga toxins (stx_1 and stx_2), intimin (*eae*), hemolysin (*hlyA*), and long polar fimbriae (*lpf1* and *lpf2*) (490, 491).

EHEC forms dense and localized microcolonies at the brush borders of fully differentiated T84 and Caco-2 cells (464, 492) (Table 2). The role of LEE gene transcription in the adhesion of EHEC has been analyzed using fully differentiated Caco-2 cells (493–496). OmpA and the long polar fimbriae of EHEC are two other factors that allow EHEC to adhere to the brush border of fully differentiated Caco-2 cells (497, 498). EHEC encodes adherence-associated loci that are involved in the initial diffuse adherence, and the intimin-Tir interaction is required for the subsequent development of EHEC microcolonies at the brush border of fully differentiated Caco-2 cells (496). The genes *yhiE*, *yhiF*, and *toxB* either up- or downregulate the expression of T3SS proteins triggering adhesion of EHEC within fully differentiated Caco-2 cells (499, 500). EHEC strains that express type 4 pili designated hemorrhagic *E. coli* pili (HCP) adhere to fully differentiated Caco-2 and T84 cells, but this adherence is not completely abolished by *hcpA* deletion, indicating that other colonization factors, e.g., intimin and *E. coli* common pilus, also contribute to EHEC adherence to these cells (501). The type V secreted autotransporter serine protease EspP and the enterohemolysin translocator EhxD of EHEC are involved in adhesion and biofilm formation in fully differentiated T84 cells (502, 503).

EHEC strains are noninvasive when they infect the apical domains of fully differentiated Caco-2 and T84 cells. The LEE-negative EHEC strain O113:H21 is internalized within fully differentiated Caco-2 cells, and intracellular bacteria are located within a membrane-bound vacuole, whereas in contrast, the EHEC strain O157:H7 remains extracellular and intimately attached to the epithelial cell surface. Curiously, EHEC strains have been found to be invasive in undifferentiated HCT-8 cells (206, 214, 215).

(vi) EAEC. EAEC strains are emerging as significant diarrheal pathogens in diverse population groups and are most commonly associated with pediatric diarrhea in developing countries (304, 413, 414, 504, 505). EAEC is also linked to diarrhea in adults, including AIDS patients and travelers, and has been a cause of food-borne outbreaks in industrialized countries. EAEC pathogenesis starts with adherence to the epithelial cells lining the terminal ileum and colon in an aggregative pattern, in which bacteria adhere to each other in a "stacked-brick" configuration (419) involving several different hydrophobic aggregative adherence fimbriae, including aggregative adherence fimbriae I and II (AAF/I and AAF/II), which are related to the Afa/Dr family of adhesins (505). Some strains of EAEC may then produce cytotoxins, including the enteroaggregative *E. coli* ST (EAST1), a partial homologue of the ETEC STa toxin, and *Shigella* enterotoxin 1 (ShET1) (505). Moreover, several EAEC strains produce toxins known as Pet (plasmid-encoded toxin) and Pic (protein involved in intestinal colonization) that belong to the subfamily of serine protease autotransporters of *Enterobacteriaceae* (SPATE) toxins (306, 506), which are secreted via the type V secretion pathway (280).

Nataro et al. (507) first reported the adhesion of EAEC at the brush borders of fully differentiated T84 and Caco-2 cells (Fig. 8A and Table 2). EAEC strain C555-91 adheres via AAF/I and AggR adhesive factors onto fully differentiated T84 cells (508). Pereira et al. (509) have reported that EAEC invades, persists, and replicates within fully differentiated T84 and Caco-2 cells for extended times.

(vii) DAEC. DAEC strains have been identified from their DA pattern on cultured epithelial cells (419, 461). DAEC is a heterogeneous group of enterovirulent *E. coli* strains (304, 412–414) including two subgroups, the non-Afa/Dr DAEC strains expressing AIDA-I adhesin (510) and the Afa/Dr DAEC strains (511, 512).

The diarrhea-causing, non-Afa/Dr DAEC strain 2787 (O126:H27) expresses AIDA-I adhesin and displays diffuse cellular adhesion to nonintestinal and nonpolarized epithelial Hep-2 and HeLa cells (510) (Table 2). AIDA-I belongs to a subgroup of autotransporter proteins that includes the TibA adhesin/invasin, associated with some ETEC strains, and the Ag43 autoaggregation factor that is found in most *E. coli* strains (513). Despite its EPEC serotype, strain 2787 is not positive for a probe of the *eae* gene (which codes for EPEC intimin), does not secrete Esp proteins, and is not able to induce the A/E lesion (514). AIDA-I adhesin contains putative binding sites (515), recognizes a 119-kDa, non-GPI, membrane-bound glycoprotein, and induces ligand-receptor clustering in nonintestinal and nonpolarized epithelial cells (516). It has been noted that despite the intestinal origin of the diarrheic AIDA-I-positive strain 2787, no information is available about the interaction and cell responses in fully differentiated Caco-2 and T84 cells. An adhesin known as CF16K that triggers DA of non-Afa/Dr DAEC in fully differentiated Caco-2 cells has been found in 9.8% of DAEC strains isolated in France (517). The CS31A adhesin and unknown adhesive factors of ET5, 3431, B6, and 0181 of human diarrheic *E. coli* strains (nonpositive for tEPEC genetic determinants) allow DA in fully differentiated Caco-2 cells (432). It is interesting to note that an association of CS31A and another adhesive factor belonging to the Afa/Dr family has been found in 70% of the diarrheic *E. coli* isolates examined.

The Afa/Dr DAEC strains have been found to be associated with urinary tract infections (UTIs) (pyelonephritis, cystitis, and asymptomatic bacteriuria) and with diarrhea in infants but not in adults (412, 518). These *E. coli* strains are generally related to the B2 phylogenetic group (519). The structural assembly genes coding for Afa/Dr DAEC adhesins have a similar organization, consisting of operons of at least five genes (511, 512). Genes A to D encode accessory proteins, and gene E encodes the major adhesin subunit AfaE-I, AfaE-II, AfaE-III, AfaE-V, DraE, or DaaE. These adhesins are assembled via the chaperone-usher pathway (260, 520). The DraE adhesin subunit expresses two separate adhesion domains; the first recognizes the human decay-accelerating factor (hDAF) at the complement control protein (CCP) 2 and 3 domains (521–524), and the second recognizes the N domains of several hCEACAMs (525–529). The physiological role of hDAF is to inhibit the complement cascade at the level of the critical C3 convertase step for the protection of normal cells from complement-mediated attack during innate activation, and in addition hDAF serves as a receptor for certain strains of pathogenic *E. coli*, *Helicobacter pylori*, and certain types of enteroviruses (530). CEACAM belonged to a group of mammalian immunoglobulin-related glycoproteins involved in cell-cell recognition and modulation of cellular processes that include the shaping of tissue architecture and neovascularization, regulation of insulin homeostasis,

T-cell proliferation, and cancers, and some membrane-bound CEACAMs have been identified as receptors for host-specific viruses and bacteria in mice and humans (531, 532). On the basis of receptor recognition or the lack thereof, two classes of DAEC strains have recently been defined: Afa/Dr DAEC and non-Afa/Dr DAEC (512). The Afa/Dr DAEC class includes *E. coli* strains harboring the AfaE-I, AfaE-II, AfaE-III, AfaE-V, Dr, Dr-II, F1845, or NFA-I adhesins, which all have the same genetic organization. The first subclass of Afa/Dr DAEC includes the AfaE-III, Dr, and F1845 adhesins, which bind to both human hDAF and human epithelial CEACAMs (hCEACAM1, hCEA, and hCEACAM6) (Afa/$Dr_{DAF/CEACAMs}$ DAEC). The second subclass of Afa/Dr DAEC includes the AfaE-I and Dr-II adhesins, which bind to hDAF but not to hCEACAMs (Afa/Dr_{DAF} DAEC).

Afa/$Dr_{DAF/CEACAMs}$ DAEC bearing the F1845 adhesin binds diffusely at the brush borders of fully differentiated Caco-2 and T84 cells by recognizing a membrane-bound receptor (Fig. 8B and Table 2). Attachment takes place during the enterocytic differentiation of the cells and parallels the apical appearance of the brush border (533, 534). *E. coli* strains bearing Dr, Afa-I, or C1845 adhesin adhere to both undifferentiated and fully differentiated Caco-2 cells (533, 535). The Dr or F1845 adhesin promotes hDAF and hCEA receptor clustering around bacteria adhering to the brush border of fully differentiated Caco-2 cells (Fig. 8C) (526).

Dr- and F1845-positive Afa/$Dr_{DAF/CEACAMs}$ DAEC enters apically infected, fully differentiated Caco-2 cells (536) to a similar extent as tEPEC. When infecting the basal domain of fully differentiated Caco-2/TC7 cells, the level of internalized Dr-positive bacteria is significantly higher than after apical infection, and the bacteria use basolateral β1 integrin for internalization. It is noteworthy that apical entry of Dr-positive bacteria is greatly enhanced after TJ opening. Lipid rafts play a role in the cell entry of Dr- and F1845-positive Afa/$Dr_{DAF/CEACAMs}$ DAEC into Caco-2/TC7 cells, since the ganglioside GM1 and VIP21/caveolin are recruited around adhering bacteria, and extraction of the membrane cholesterol with methyl-beta-cyclodextrin disorganizes the membrane lipid rafts and inhibits the cell entry of Afa/$Dr_{DAF/CEACAMs}$ DAEC (536, 537). It is important to note that it has been established that the cell entry of Afa/$Dr_{DAF/CEACAMs}$ DAEC into epithelial cells is triggered by their major adhesin subunits E (538, 539). The role of the D subunits of Afa/$Dr_{DAF/CEACAMs}$ DAEC, known as invasins (540, 541), in the entry into epithelial cells remains controversial (542–547).

Structural and Functional Injuries

Enterovirulent bacteria cause diarrhea by means of sophisticated strategies that allow them to hijack the cellular machinery to abolish or manipulate the normal transportation of nutrients and the water balance by altering ion channels and/or exchangers and water channels (304, 309, 548) (Fig. 2). The intestinal brush border consists of tightly packed, uniform, apical microvilli. The microvillus membrane is the first cell barrier encountered by enterovirulent bacteria infecting the host from the luminal intestinal compartment. The brush border membrane is supported by a cytoskeleton composed of actin microfilaments and several actin-binding proteins, including the F-actin cross-linkers, villin and fimbrin, fodrin, and plastin-1, and a protein complex composed of brush border myosin I and II associated with calmodulin, which connect the F-actin bundles to the plasma membrane (85–87). The microvillus membrane of mature enterocytes contains hydrolases (108), transporters involved in nutrient uptake (549), hexose transporters (550), and channels and transporters acting in ion transport (128, 129, 551). Elegant studies have revealed the molecular mechanisms by which enterovirulent bacteria, via the action of their T3SS-translocated bacterial effectors or secreted and endocytosed cytotonic toxins, affect the functionality of membrane-associated proteins involved in the control of nutrient and ion intestinal transports (551) and NHE antiporters localized at the membrane of the brush border or basal membrane (128, 129, 241). It is noteworthy that the role of the enteric nervous system in the enterovirulent bacterium-induced modifications of the localization or activity of structural and functional cell proteins controlling the intestinal transport of nutrients and ions is now beginning to be investigated. Mainly as a result of *in vitro* experiments using appropriate cultured human intestinal cell lines representing models of human enterocytes or colonic cells, we can briefly summarize that diarrhea promoted by enterovirulent *E. coli* is the result of ETEC adhering to small bowel enterocytes and inducing watery diarrhea by secreting heat-labile and/or heat-stable enterotoxins. tEPEC, aEPEC, and EHEC all have the ability to form A/E lesions, and this is the main pathogenic mechanism in both groups (287, 303, 304, 412, 413, 485). This structural lesion results from intimate bacterial adherence, local microvillus effacement and, the accumulation of polymerized actin and other elements of the cytoskeleton underneath adherent bacteria, which form pedestal-like structures. EHEC also produces Stxs (307). EAEC produces secretory enterotoxins and cytotoxins. EIEC invades colonic epithelial cells, resides within intracellular vacuoles, lyses the phagosome, moves within the cell cytoplasm, and finally uses cell-to-cell spread for penetrating neighboring cells. Afa/Dr DAEC does not form these A/E lesions, but it too triggers a loss of microvilli resulting from disorganization of the brush border cytoskeleton that promotes the shedding of microvilli. In all cases, the loss of microvilli results from a dramatic disappearance of the membrane-anchored proteins that control absorption/secretion functions and leads to dramatic defects in nutrient and electrolyte transportation.

Listeria. LLO induces an increase in short-circuit current and chloride secretion in fully differentiated HT-29/B6 cells (552).

V. cholerae. *V. cholerae* induces functional injuries at the brush border of enterocytes without affecting the structure of the microvilli. *V. cholerae* promotes severe dysfunctions in enterocytes as the result of the cytotoxic or cytotonic actions of toxins (Table 3). CT enters into fully differentiated T84 cells via apical but not basolateral membranes (553). Binding of toxin B subunits to ganglioside GM1 localized in the membranes of microvilli of fully differentiated Caco-2 and T84 cells is followed by the translocation of the enzymatically active A subunit across the membrane and then retrograde traffic into the cells (554, 555) and subsequent activation of the cAMP adenylate cyclase located on the basolateral membrane (556–560). Fully differentiated, mucus-secreting HT-29-18 N2 cells have been also used to investigate the binding of CT to human intestinal cells (561). Antibodies directed against CT produce a concentration-dependent blockade of CT-induced Cl^- secretion and completely inhibit binding of CT to apical cell membranes of fully differentiated T84 cells (562). The enzymatically active A subunit of CT, which contains the ER retention signal KDEL, interacts directly with endogenous KDEL receptors in fully differentiated T84 cells and undergoes retrograde movement through the Golgi cisternae and endoplasmic reticulum in order to produce the biological activity (563). In *V. cholerae*-in-

TABLE 3 Overview of functional injuries in fully differentiated human colon cancer cell lines

Pathogen	Cell line	Pathogenicity island, virulence factor, or cellular protein	Effect	Reference(s)
Listeria	HT-29/B6	LLO	Increase of short-circuit current, chloride secretion, and transepithelial flux	552
Vibrio cholerae	Caco-2		Activation of SLC7A11 and downregulation of SLC6A14 transporters, AQP10, Mg^{2+} channel TRPM6, SERT, SVCT1, ZnT4	134
	Caco-2	Cholera toxin	Increased expression of decay-accelerating factor and α1-antichymotrypsin	565
	Caco-2	Cholera toxin	Downregulation of AMPs LL-37 and HBD-1	574
ETEC	Caco-2	LT	Inhibition of PEPT1	573
	Caco-2, T84	LT	Protein kinase A-, Erk1/2-, and COX-2-dependent downregulation of cathelicidin hCAP-18/LL-37 and HBD-1	574
	Caco-2 BBe	ST	Activation of exocytosis of syntaxin 3 and CFTR	601
	Caco-2	ST	Stimulation of cGMP-chloride secretion	595–598
	T84	ST	Increase of net secretory transepithelial vol flux, short-circuit current, and net secretory Cl flux	599
	T84	ST	F-actin cytoskeleton-dependent activation of $Na^+/K^+/2Cl^-$ cotransport and Cl^- secretion	600
	Caco-2 BBe	LT, Sta	Activation of exocytosis of syntaxin 3 and CFTR	601
EIEC	HT-29.cl19A, Caco-2	Undetermined	Increase of chloride secretion	672
tEPEC	Caco-2	EspA, EspB, EspD	Roles in the increase in electrolyte transport	611, 612
	T84	Independently of T3SS and A/E lesion	Decrease of cAMP-dependent ion secretion by downregulation of the brush border-associated CFTR	616
	Caco-2, T84	T3SS dependent	Observation of changes in activities of NHE isoforms (increase for apical NHE2, decrease for apical NHE3, and increase for basolateral NHE1	613
	Caco-2	Undetermined	Ca^{2+}-, PKC-α- and PKC-ε-dependent increase of NHE2 activity	615
	Caco-2	Undetermined	Inactivation of Na^+/glucose cotransporter SGLT-1	606
	Caco-2	Map	Proteolysis of NHERF1	617
			Inhibition of the apical MCT1	618
	Caco-2	T3SS dependent	Inhibition of apical Cl^-/OH^- exchange activity	620
			Inhibition of vitamin B_1 uptake	621
	Caco-2, T84	EspG, EspG2	Decrease of apical activity of SERT	622
	Caco-2	T3SS dependent	Cdx2-dependent upregulation and increase of activity of hPEPT1 transporter	623
	Caco-2, HT-29.cl19A	Undetermined	Roles in inhibitory effect on apical sodium-dependent bile acid transporter	624
EHEC	Caco-2	T3SS, Stx1	Increases apical galectin-3 expression and secretion, impairs trafficking, and promotes apical protein mistargeting of villin, DPP IV, and NHE-2	639, 640
EAEC	T84	EAST1	Increase in short-circuit current and net ion transport	646
Afa/$Dr_{DAF/CEACAMs}$ DAEC	Caco-2	Dr, F1845	Cytoskeleton-dependent loss of expression of brush border-associated SI, DPP IV, glucose transporter SGLT1, and fructose transporter GLUT5 and abolition of sucrase and DPP IV enzyme activities	649, 650
	Caco-2	Unknown factor	Blockade of SI and DPP IV biosynthesis without affecting mRNA levels and enzyme stability	650

fected fully differentiated Caco-2 cells, two amino acid transporters, SLC7A11 and SLC6A14, are upregulated, and five transporters (AQP10, a member of the water channel family of transmembrane proteins that transport water as well as glycerol and other solutes of small molecules [24, 25, 564], the Mg^{2+} channel transient receptor potential cation channel subfamily M member 6 [TRPM6], the serotonin transporter [SERT] controlling the uptake of serotonin that is involved in intestinal absorption and secretion of electrolytes and fluids, the vitamin C transporter [SVCT1], and the zinc transporter [ZnT4]) are all downregulated,

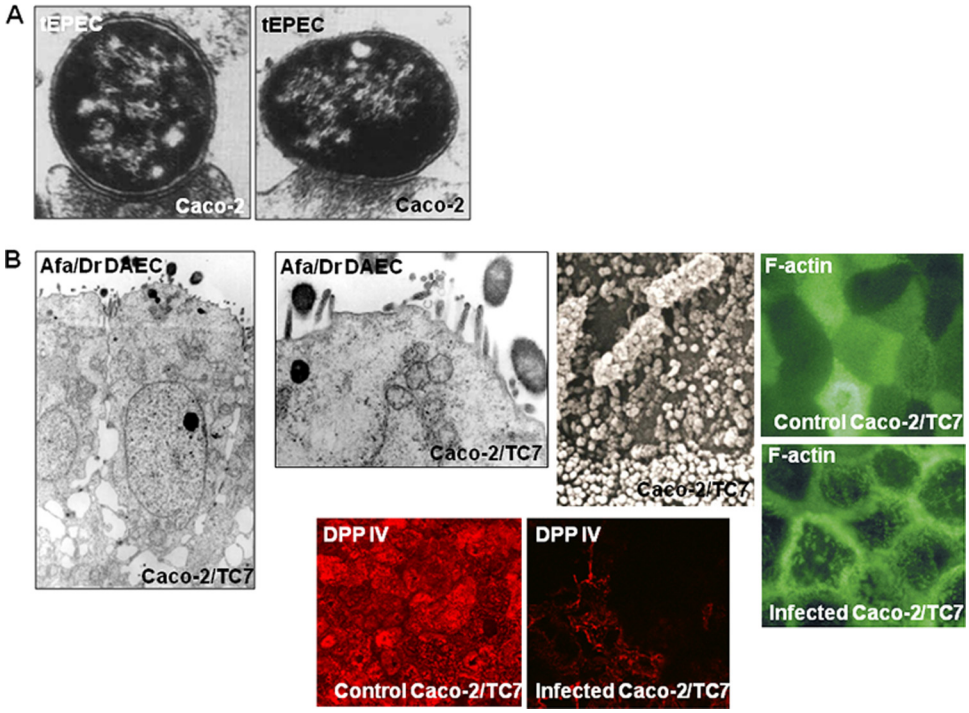

FIG 9 Structural and functional injuries caused by enterovirulent bacteria adhering to the brush border of cultured human fully differentiated colon cancer cells. (A) Transmission electron microscopy micrographs show A/E lesions of microvilli and pedestal formation by tEPEC adhering to the brush border of fully differentiated parental Caco-2 cells. The left micrograph shows tEPEC starting the effacement of a microvillus. The right micrograph shows the achieved effacement of a microvillus by tEPEC and the formed pedestal. (B) Transmission and scanning electron microscopy micrographs show the disappearance of the brush border in fully differentiated Caco-2/TC7 clone cells infected with Afa/Dr $DAEC_{DAF/CEACAM}$. Immunofluorescence labeling and confocal laser scanning microscopy examination (*x-y* section) show the loss of apical F actin and brush border-associated DPP IV hydrolase in fully differentiated Caco-2/TC7 clone cells infected with Afa/Dr $DAEC_{DAF/CEACAM}$. (The two tEPEC micrographs in panel A are reprinted from reference 467 with permission from the International Society of Differentiation; the two Afa/Dr DAEC micrographs in panel B are reprinted from reference 647.)

mimicking the situation in infected patients (134). The increased expression of DAF and SERPINA3 protein (α1-antichymotrypsin) in CT-stimulated fully differentiated Caco-2 cells has been also found in the intestinal mucosa during acute cholera (565).

Enterovirulent *E. coli*. (i) ETEC. To cause diarrhea, ETEC elaborates and delivers LT and/or ST, which elicit watery, cholera-like diarrhea (Table 3). Using fully differentiated Caco-2 and T84 cells, it has been demonstrated that ETEC strains use their adhesive factors to deliver toxins efficiently to the brush border expressing the receptors for ETEC enterotoxins (566, 567). LT, like the *Vibrio cholerae* enterotoxin, stimulates the cAMP adenylate cyclase in fully differentiated Caco-2 cells (568–572). In contrast to CT (560), LT is not localized within caveola-like detergent-insoluble membranes of fully differentiated T84 cells (567). Apical entry, but not basolateral entry, of LT into fully differentiated T84 cells leads to the production and activation of a toxin A through the proteolytic action of a serine protease (553). In fully differentiated Caco-2 cells, LT inhibits the activity of PEPT1 (573). LT transcriptionally downregulates two human antimicrobial peptides, cathelicidin hCAP-18/LL-37 and HBD-1, by activating several intracellular signaling pathways involving protein kinase A, Erk1/2, and cyclooxygenase 2 (COX-2) downstream of cAMP accumulation and inducible cAMP early repressor in fully differentiated HT-29, Caco-2, and T84 cell lines (574).

ST stimulates the cGMP adenylate cyclase in fully differentiated T84 cells (575–577) (Table 3). After binding to fully differentiated Caco-2 cells, ST leads to receptor activation followed by the production of high intracellular levels of cGMP (578, 579). The characteristics of ST B bound to the cell membrane (580–583), the characterization and partial purification of the receptor of ST (584), the regulation of the receptor (585–593), and the internalization of the receptor (594) have been extensively investigated in fully differentiated T84 cells. ST induces cGMP-chloride secretion in fully differentiated Caco-2 cells (595–598). ST increases the net secretory transepithelial volume flux, short-circuit current values, and net secretory Cl flux in fully differentiated T84 cells (599). $Na^+/K^+/2Cl^-$ cotransport and Cl^- secretion induced by ST have been found to be dependent on the organization of the F-actin cytoskeleton in fully differentiated T84 cells (600). Both LT and STa activate the exocytosis of syntaxin 3, an intestine-specific soluble *N*-ethylmaleimide-sensitive factor attachment receptor, and a functional CFTR chloride channel that colocalize at the apical domain of fully differentiated Caco-2 BBe clone cells (601). It is noteworthy that in fully differentiated T84 cells, STa and guanylin did not increase paracellular permeability, but STa elicited a slight reduction in TER whereas guanylin did not (602).

(ii) EPEC. The main intestinal cell lesion promoted by tEPEC and aEPEC strains is the A/E histopathology, first observed by Knutton et al. (464) in fully differentiated Caco-2 cells, which is characterized by effacement of brush border microvilli after intimate adherence of the bacterium to the epithelial cell membrane (Fig. 9A and Table 4). The microvillous lesion results from the

TABLE 4 Overview of structural injuries at the brush borders of fully differentiated human colon cancer cell lines

Pathogen	Cell line	Pathogenicity island, virulence factor, or cellular protein	Effect	Reference(s)
Salmonella	Caco–2	Undetermined	Reorganization of the F–actin cytoskeleton, disruption of the brush border with elongation of the microvilli, and membrane remodeling for cell entry	374–377, 389
	Caco-2	SopE2, SopB, SipA	Reorganization of the F-actin cytoskeleton and membrane remodeling for cell entry	390
tEPEC	Caco-2	Undetermined	A/E microvillus lesion and presence of concentrated F actin at the pedestal like structure	464
	Caco-2	Intimin, T3SS effectors	Identification for A/E lesion	603, 604
	Caco-2	EspA, EspB, EspD	Identification for A/E lesion	605
	Caco-2	Undetermined	Role of Eps15 and epsin1 in clathrin-dependent pedestal formation	608
	Caco-2	Map, EspF, Tir, intimin	Microvillus effacement	606
	Caco-2/TC7	EspF	Microvillus effacement dependent on its N-WASP binding motif	93
aEPEC	Caco-2	Undetermined	A/E lesion without F-actin condensation	610
EHEC	Caco2, T84	Undetermined	A/E lesion	464, 492
EAEC	Caco-2, T84	Undetermined	Loss of microvilli and subnuclear vacuolization only in T84 cells	507, 644, 645
	T84	Pet	No structural cytotoxic effect	645
Afa/$Dr_{DAF/CEACAMs}$ DAEC	Caco	F1845	Disassembly of the apical F-actin cytoskeleton in turn affecting the structural organization of the brush border and the loss of microvilli	647, 649
	Caco-2/TC7	Dr, F1845	Disassembly of F-actin cytoskeleton by NETs produced by infected PMNL-like human myeloid cell line PLB-985	813
	Caco-2/TC7	Dr, F1845	Disassembly of apical F-actin cytoskeleton	813
	T84	F1845	Induction of HIF-1α leads to loss of E-cadherin and cytokeratin 18 and a rise in fibronectin indicating the induction of a cell epithelial-to-mesenchymal transition-like phenotype	820

action of T3SS in translocating into the cells an assortment of effector proteins, encoded both within and outside the LEE, which generate various coordinated cell signaling events (287). The LEE is organized into 5 operons (*LEE1* to *LEE5*), where *LEE1* to *LEE3* encode T3SS proteins and the LEE-encoded regulator, *LEE4* encodes the secreted proteins that form the external part of the T3SS used to translocate effector proteins into the host cell, and *LEE5* encodes the adhesin intimin and its translocated receptor, Tir. Membrane-inserted Tir is phosphorylated at the C-terminal domain, where it recruits the cellular protein Nck and activates N-WASP, leading to Arp2/3 complex-mediated actin polymerization. Knutton et al. (464) observed the presence of concentrated cellular F actin at the sites of attachment in fully differentiated Caco-2 cells and reported that after effacement of the microvillus, tEPEC bacteria are seen localized on a pedestal-like structure. tEPEC BFP, adhesin intimin, and several translocated T3SS effectors have been identified as intimately adhering to fully differentiated Caco-2 cells (603, 604). tEPEC T3SS effectors EspA, EspB, and EspD have been shown to be required for the formation of the A/E lesions in fully differentiated Caco-2 cells (605). tEPEC-induced effacement of microvilli in fully differentiated Caco-2 cells requires the cooperative action of T3SS effectors Map (mitochondrion-associated protein), EspF, and Tir as well as intimin (606). Microvillus effacement activity of the tEPEC protein EspF in the Caco-2/TC7 cells was dependent on its N-WASP binding motif (93). EspB binds to myosin and blocks its interaction with actin in fully differentiated Caco-2 cells (607). tEPEC uses the clathrin-coated pit components Eps15 and epsin1, but not adaptor protein 2 (AP-2), in clathrin-dependent pedestal formation in fully differentiated Caco-2 cells (608). tEPEC infection of clone Caco-2/B7 results in a T3SS-dependent recruitment of ZO-1 at the actin-rich pedestals (92). Moreover, tEPEC recruits and subsequently sequesters cell surface nucleolin around the bacteria present in tEPEC microcolonies in fully differentiated Caco-2 cells, a phenomenon that is unrelated to tEPEC-induced pedestal formation or microvillus effacement but apparently is related to the tEPEC-induced disruption of the epithelial barrier function (609).

aEPEC strains have been shown to induce A/E lesions on the microvilli in fully differentiated Caco-2 cells without F-actin condensation (610) (Table 4).

As a consequence of the tEPEC-induced A/E lesions described above, which lead to the disappearance of the brush border microvilli, but also independently of A/E lesions, functional injuries develop in tEPEC-infected fully differentiated intestinal cells (Table 3). tEPEC stimulates electrolyte transport in fully differentiated Caco-2 cell monolayers through the action of the T3SS-translocated effectors EspA, EspB, and EspD (611, 612). tEPEC affects the NHE isoforms, the major mediators of Na^+ absorption, increasing the activity of apical isoform NHE2, decreasing that of apical NHE3, and increasing that of the basolateral isoform NHE1 via a T3SS-dependent mechanism in fully differentiated Caco-2 and T84 cells (613). There was no change in the basolateral K^+ channel or $Na^+/K^+/2Cl^-$ cotransport activity in tEPEC-infected fully differentiated T84 cells (614). Signal transduction cascades involving Ca^{2+}, as well as protein kinase C alpha (PKC-α) and PKC-ε, occur independently of A/E lesions but require tEPEC adhesion and are responsible for the increase in NHE2 activity in fully differentiated Caco-2 cells (615). Independently of flagellin,

T3SS, and A/E lesions and independently of loss of TER and TJ alteration, tEPEC decreases the cAMP-dependent ion secretion in fully differentiated T84 cells by promoting the downregulation of the brush border-associated CFTR involved in the control of chloride secretion (616). tEPEC rapidly inactivates the Na^+/glucose cotransporter SGLT1 in fully differentiated Caco-2 cells by multiple mechanisms that are either dependent on or independent of the A/E lesion (606). The T3SS effector Map induces brush border elongation in fully differentiated Caco-2 cells and proteolysis of Na^+/H^+ exchanger regulatory factor 1 (NHERF1) (617). T3SS-dependent inhibition of the short-chain fatty acid absorption mediated by apical MCT1 develops in tEPEC-infected fully differentiated Caco-2 cells (618). The tEPEC T3SS effectors EspG and EspG2 are involved in the inhibition of apical anion exchanger DRA, functionally coupled to CFTR in the upper gastrointestinal tract to mediate chloride and bicarbonate secretion and to NHE3 in the lower gastrointestinal tract to mediate electroneutral NaCl absorption (619), corresponding to a decreased $Cl^-/HCO_3/OH^-$ exchange activity in fully differentiated Caco-2 and T84 cells (620). tEPEC T3SS-dependent inhibition of the uptake of the water-soluble vitamin B_1 (thiamine) has been observed in fully differentiated Caco-2 cells (621). The apical activity of SERT is decreased following tEPEC infection of fully differentiated Caco-2 cells in a T3SS-dependent manner, but its expression at the brush border is unaffected (622). Intimate attachment of tEPEC to fully differentiated HT29-Cl.19A cells is followed by increased hPet1 expression and activity by activation of the transcription factor Cdx2 (623). Deletion of the *escN*, *espA*, *espB*, and *espD* genes, which encode parts of T3SS, and the gene encoding BFP revealed that both early and intimate tEPEC attachment are needed for the EPEC inhibitory effect on the apical sodium-dependent bile acid transporter to occur in fully differentiated Caco-2 cells (624). It is interesting to note that apical AQP2 and basolateral AQP3 in colonocytes are mislocalized from their normal location along cell membranes to the cell cytoplasm in mice infected with the A/E pathogen *C. rodentium*, indicating a contribution to the diarrhea caused by this organism (625). The mechanisms by which tEPEC strains affect the distribution and activity of AQPs remain to be investigated in cultured, fully differentiated intestinal cells.

EspC is an tEPEC toxin belonging to the SPATE toxin family (506), which are all secreted via the type V secretion pathway (280) (Table 3). EspC toxin acts within the cytosol of epithelial cells to disrupt the architecture of the F-actin cytoskeleton (626–628). An irreversible cell cycle arrest at the G_2/M transition and sustained inhibitory phosphorylation of the mitosis inducer CDK1 have been found to be triggered by the tEPEC cycle-inhibiting factor (Cif) (629), which belongs to the cyclomodulin family of bacterial toxins and effector proteins (630).

(iii) EHEC. By forming dense and localized microcolonies at the brush borders of fully differentiated T84 and Caco-2 cells, EHEC induces the classical A/E brush border lesion (464, 492) (Table 4). Like tEPEC, EHEC promotes the A/E histopathology that results from the LEE pathogenicity island that encodes T3SS and effector proteins homologous to those produced by tEPEC (287, 631). Effacement of the brush border microvilli of intestinal cells follows intimate adherence between the bacterium and the epithelial cell membrane, accumulation of polymerized actin beneath the adhering bacteria, and effacement of microvilli, resulting in the bacteria being localized on a pedestal-like structure. However, there is a significant divergence between the structures of EHEC Tir effector proteins and those of tEPEC and also between the resultant pathogenic mechanisms utilized for EHEC-induced host cell actin polymerization (287, 305, 631). *E. coli* strains expressing Stxs but lacking the LEE have been shown to adhere at the brush border of fully differentiated Caco-2 cells (632), to induce F-actin cytoskeleton rearrangements, and to impair epithelial barrier function and ion transport in fully differentiated T84 cells (633) (Table 4).

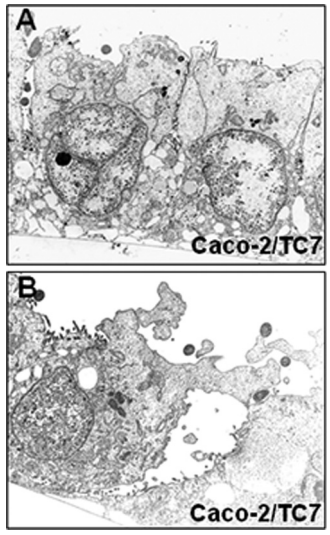

FIG 10 Structural injuries and signs of cell death in fully differentiated Caco-2/TC7 clone cells infected with a Shiga toxin-positive *E. coli* strain. (A) Transmission electron microscopy micrograph of infected cells at an early time of infection, showing the loss of brush border and the opening of the junctional domain without a modification of the polarized organization of the cells forming a monolayer. (B) Transmission electron microscopy micrograph of infected cells at a late time of infection, showing the alteration of cell polarization, the disorganization of the apical domain, the detachment of cell debris, the appearance of a large disjunction at the junctional domain, and the cell detachment at the basal domain, indicating cell death.

Stxs are classical A_1B_5 toxins composed of two subunits: a pentameric array of binding subunits and a single active subunit (634) (Table 4). There are two main Stx families, Stx1 and Stx2. The Stx1 family consists of Stx1, Stx1c, and Stx1d, and the Stx2 group consists of Stx2, Stx2c, Stx2c2, Stx2d, Stx2e, and Stx2f. EHEC adhering at the apical surface of fully differentiated T84 cells induces Src-dependent macropinocytosis that allows the delivered Stx1 to penetrate and traffic within the cells (635–638) (Table 3). EHEC expressing Stx1 causes galectin 3 depletion in fully differentiated T84 cells by increasing the expression and secretion of apical galectin 3, which in turn impairs trafficking and apical protein mistargeting of several brush border structural proteins and transporters, including villin, DPP IV, and NHE2 (639, 640). Stx causes the inhibition of protein synthesis and apoptosis in fully differentiated Caco-2 cells (Fig. 10) but not in T84 cells (641). Stx1 (642, 643) and Stx2 (643) both exhibit cytotoxic activity and apoptosis in fully differentiated Caco-2 cells, since they induce p53/ATM-dependent nuclear condensation, fragmentation of chromosomal DNA, and cleavage of PARP (642).

(iv) EAEC. The cytotoxic effects of EAEC strains have been investigated mainly using polarized, fully differentiated colon cancer T84 cells. EAEC strain 042, which adheres to the brush borders of cultured, fully differentiated human T84 and Caco-2 cells, pro-

motes apical membrane damage characterized by the loss of microvilli and subnuclear vacuolization (507, 644, 645) (Table 4). It is noteworthy that EAEC have been observed inside fully differentiated T84 cells (644).

Exposure of fully differentiated T84 cell monolayers mounted in Ussing flux chambers to purified enteroaggregative heat-stable toxin 1 (EAST1) results in an increase in the short-circuit current, a sensitive measure of net ion transport (646). The Pet toxin secreted by EAEC belongs to the SPATE family of toxins (306, 506). Pet disrupts the architecture of the F-actin cytoskeleton of epithelial cells by causing the cleavage of the actin-binding protein fodrin (626–628) (Table 4). The Pic (protein involved in colonization) toxin is a second SPATE toxin produced by EAEC that does not damage epithelial cells, cleave fodrin, or degrade host defense proteins embedded in the mucus layer but which does display mucinolytic activity, possibly enabling EAEC to penetrate the intestinal mucus layer during EAEC colonization (626). The cytotoxic effects of Pet and Pic have been investigated mainly using nonintestinal and nonpolarized epithelial Hep-2 cells. The cytotoxic effect of EAEC investigated in fully differentiated T84 cells is clearly independent of Pet (645).

(v) Afa/Dr DAEC. Bernet-Camard et al. (647) were the first to report that in fully differentiated Caco-2 cells infected with a wild-type, F1845-positive Afa/$Dr_{DAF/CEACAMs}$ DAEC strain, the recognition of hDAF by the adhesin is followed by the disassembly of the apical F-actin cytoskeleton, which in turn dramatically alters the structural organization of the brush border (Fig. 9B and Table 4). When the bacteria come into contact with the brush border, this results in the elongation of the microvilli and the formation of tip microvillus vesicles that after detaching from the cells remain attached to the infecting bacteria. At a late time postinfection, the brush border of the infected cells disappears (Fig. 9B). The disappearance of the brush border observed in Afa/$Dr_{DAF/CEACAMs}$ DAEC-infected fully differentiated Caco-2 cells at a late time postinfection resembles the disappearance of the brush border observed in tEPEC-, aEPEC-, or EHEC-infected fully differentiated Caco-2 cells. However, the Afa/$Dr_{DAF/CEACAMs}$ DAEC-induced disappearance of the brush border results from mechanisms that differ from those of tEPEC, aEPEC, and EHEC, since Afa/$Dr_{DAF/CEACAMs}$ DEAC strains do not express LEE leading to the A/E lesion of microvilli. Afa/$Dr_{DAF/CEACAMs}$ DAEC expressing Dr-II adhesin promotes apical cytoskeleton rearrangements and, as the result of the presence of a functional hemolysin, promotes cell apoptosis and cell lysis in fully differentiated Caco-2 cells (648). Accompanying the Dr or F1845 adhesin-induced apical F-actin cytoskeleton disassembly in fully differentiated Caco-2 cells, the distribution of brush border-associated functional proteins SI, DPP IV, glucose transporter SGLT1, and fructose transporter GLUT5 is dramatically altered (649), and as a consequence, brush border sucrase and DPP IV enzyme activities are abolished (650) (Fig. 9B and Table 3). In parallel, the fully differentiated Caco-2 cells infected with Afa/$Dr_{DAF/CEACAMs}$ DAEC expressing Dr or F1845 adhesin display a blockade of the biosynthesis of SI and DPP IV without mRNA levels or enzyme stability being affected (650). Interestingly, when the cells are infected with recombinant *E. coli* strains expressing the Dr or F1845 adhesin, no decrease in sucrase or DPP IV enzyme activities and no inhibition of enzyme biosynthesis are observed, indicating that a pathogenic factor(s) other than the Afa/$Dr_{DAF/CEACAMs}$ adhesins operates in Afa/$Dr_{DAF/CEACAMs}$ DAEC.

Structural and Functional Injuries at the Junctional Domain

TJs are positioned most apically in the junctional domain of epithelial cells. TJs regulated in response to physiological and immunological stimuli are the primary cellular determinant of intestinal epithelial barrier function (23, 651, 652). TJs are also involved in mucosal immune responses (23, 651, 652). TJ organization results from the interaction of transmembrane proteins, including the structural ZO proteins linked to the actin filament cytoskeleton, the structural/functional occludin, and the functional claudins (23, 651, 652). TJs are a highly regulated cell area in which the Ca^{2+}-calmodulin-dependent serine-threonine protein kinases myosin light chain kinase (MLCK) and PKC-α play an essential role for the functional regulation (23, 651, 652). Some enterovirulent bacteria have developed sophisticated strategies for structurally and functionally altering the TJs by delocalizing ZO proteins, occludin, and claudins but also other cell membrane-anchored proteins such as aquaporins (23, 309, 653). Some recent studies have shown that an increase in paracellular permeability can occur both with and without changes to TER. Disruption of the structure and functions of TJs by enterovirulent bacterial effectors and toxins that lead to deregulation of the paracellular passage of solutes is one of the causes of diarrheal disease (653) (Table 5). Moreover, enterovirulent bacteria, which are unable to cross the cell membrane at the brush border but are able to enter through the basal membrane, have acquired the capacity to open the TJs by means of their translocated effectors or secreted toxins and thus to penetrate into the intestinal polarized epithelial cells through the junctional domain.

Shigella. *S. flexneri* serotype 2a adhering apically onto fully differentiated T84 cells secretes bacterial products that have the ability to alter the TJs by inducing the delocalization of ZO-1, occludin, and claudin-1 from membrane lipid rafts and the dephosphorylation of occludin (654). In fully differentiated Caco-2 cells cocultured with enteric glial cells, which function as important regulators of intestinal epithelial barrier functions, and in *ex vivo* cultured human colonic mucosa, Flamant et al. (655) observed a reduced deleterious effect of *S. flexneri* on the TJs, indicating that the enteric glial cells have a protective effect against the lesions at the intestinal barrier caused by an invasive pathogen.

Listeria. LLO induces a loss of TER and an increase in transepithelial flux in fully differentiated HT-29/B6 cells (552).

C. jejuni. *C. jejuni* causes no changes in short-circuit currents when fully differentiated Caco-2 cell monolayers mounted in Ussing chambers are infected, but dome formation, a marker of fluid transport across the monolayer, is reduced, and this is accompanied by a decrease in TER correlating with a change in the distribution of the TJ-associated occludin (656) (Table 5). Apical infection of fully differentiated T84 monolayers causes a decrease in TER accompanied by the redistribution of the TJ-associated occludin from an intercellular to an intracellular location (657). When infecting the basolateral domain, *C. jejuni* causes a more rapid decrease in TER but a comparable redistribution of TJ proteins (657). Internalization of *C. jejuni* into fully differentiated T84 cells through a phosphoinositide 3-kinase (PI3K)-dependent mechanism is accompanied by a reduction of TER (658).

***Salmonella* spp.** Fully differentiated Caco-2 cells grown on permeable filters and infected apically with either *S.* Choleraesuis or *S.* Typhimurium show a loss of TER (377) (Table 5). *S.* Typhimurium delocalizes the TJ-associated ZO-1 and occludin proteins in

TABLE 5 Overview of structural and functional injuries at the junctional domains of fully differentiated human colon cancer cell lines

Pathogen	Cell line	Virulence factor	Effect	Reference(s)
Shigella	T84	Undetermined	Loss of ZO-1, occludin, and claudin-1 from membrane lipid rafts and dephosphorylation of occludin	654
Listeria	HT-29/B6	LLO	Loss of TER	552
Campylobacter	Caco-2	Undetermined	Reduction of dome formation, loss of TER, and alteration of occludin distribution	656
	T84	Undetermined	Loss of TER and alteration of TJ-associated protein distribution	657
Salmonella	Caco-2	Undetermined	Loss of TER	377
	Caco-2/TC7	SopB, SopE, SopE2, SipA	Rho GTPase-dependent disruption of TJs and delocalization of ZO-1 and occludin	659
Vibrio cholerae	T84	Undetermined	Loss of TER	668.
	T84	RTX toxin	Loss of TER	671
	T84	Cholera toxin	Increase of Isc, activates Cl^-/HCO_3^- transport	667
	T84	Protease PrtV and hemolytic exotoxin VCC	Increase in paracellular permeability	760
	Caco-2	ZOT	Increase in paracellular permeability	662
	Caco-2	ZOT	Delocalization of occludin and ZO-1 and loss of TER	663
	Caco-2	ZOT	Activation of PAR2 for disassembly of TJ proteins	664
EIEC	HT-29.cl19A, Caco-2, T84	Undetermined	Loss of TER, rearrangements of ZO-1 and occludin, and phosphorylation of occludin	672
	T84	Undetermined	Loss of TER, increase of paracellular flux, and redistribution of ZO-1 protein	455
tEPEC	Caco-2	Undetermined	Loss of TER	674-676
	T84	Undetermined	Activation of myosin light chain kinase (MLC20) for loss of TER	679
	T84	Tir	Role in loss of TER	680, 681
	Caco-2	EspG, Orf3	EspG alone in cells is without an effect on TER, whereas EspG together with Orf3 decreases TER	691
	T84	EspF		682
	Caco-2	Undetermined	Rearrangements of ZO-1, occludin, and claudin-1 proteins accompanying loss in TER; loss of fence function	688
	Caco-2	NleA	Loss of TER results of rearrangement in distribution of TJ-associated ZO-1 and occludin and the delocalization of occludin but not claudin-1 and -3 from lipid rafts	686, 687
	Caco-2	EspF, Map	TJ disruption through inhibition of COPII-dependent host cell protein trafficking	684, 685
	Caco-2	EspG, EspG2	Disruption of TJs and increase the paracellular movement of molecules	690
	T84	Undertermined	Activation of the cysteine protease calpain leads to a loss of TER and a rapid cell loss and necrosis, a phenomenon increased in the absence of Tir	683
	Caco-2	EspG, Orf3	Alteration of TJ fence function delocalizing basolateral proteins, including β1-integrin and Na^+/K^+ ATPase to the apical membrane	691
	Caco-2, T84	EspG (EspG-α and EspG-β) and EspG1 and -2	Microtubule disruption and loss of TER	692, 693
EHEC	T84	Undetermined	Loss of TER, increase of fluid transepithelial passage, reduced expression of TJ ZO-1, occludin, and claudin-2 but not claudin-1 or claudin-4	697, 698
EAEC	T84		Loss of TER, delocalization of TJ claudin-1 and occludin	699
$Afa/Dr_{DAF/CEACAMs}$ DAEC	Caco-2/TC7	F1845 adhesin independent	Increase of paracellular permeability without loss of TER and with ZO-1 and occludin delocalization	700
	Caco-2/TC7	Sat	Rearrangements of TJ ZO-1, ZO-3, and occludin but not of claudin-1, changes in distribution of ZO-1, ZO-3, and occludin within lipid rafts, increase of formation of fluid domes without affecting TER	701

fully differentiated Caco-2/TC7 clone cells (659). SopB, SopE, SopE2, and SipA are the T3SS-1-secreted effectors responsible for the *S.* Typhimurium-induced disruption of TJ structure and function by stimulating host cell Rho family GTPases (659).

V. cholerae. The characterization of the epithelial cell receptor for Zot conducted in fully differentiated Caco-2 cells has revealed a 66-kDa membrane receptor protein localized at the intercellular contacts and that Zot binding to its receptor requires a sequence that spans between amino acids (aa) 118 and 299 (660, 661). Consistent with the localization of the Zot receptor at the cellular junction, the 12-kDa fragment of Zot, deltaG, enhanced the paracellular permeability in fully differentiated Caco-2 cell monolayers (662). Consistent with this, the Zot C-terminal domain causes the delocalization of occludin and ZO-1 from the TJs of fully differentiated Caco-2 cell monolayers, without causing F-actin reorganization or any change in TER (663) (Table 5). Moreover, the active Zot domain (aa 288 to 293) increases ZO-1 and myosin 1C serine/threonine phosphorylation, altering interaction between ZO-1 and its binding partners, which in turn induces TJ disassembly through PAR2 activation (664). It was noted that zonulin, a eukaryotic protein structurally similar to Zot (404), induced TJ disassembly, resulting in the delocalization of the TJ-associated ZO-1 protein in fully differentiated Caco-2 cells (665). It is noteworthy that opening of the TJs by Zot is a new approach tested for the safe delivery of therapeutic agents (666). By using Ace, *V. cholerae* stimulates a rapid increase in Isc and the activation of Cl^-/HCO_3^- transport in fully differentiated T84 cells as the result of changes in intracellular Ca^{2+}, but this is not associated with any increase in intracellular cyclic nucleotides (667). Decreased TER has been also observed in fully differentiated T84 cells as a result of *V. cholerae* protease activity (668) (Table 5). RTX toxin (669), which is produced by El Tor and O139 strains but not by classical strains and which depolymerizes cellular actin (670), causes a loss of TER in fully differentiated T84 cells when added to either the apical or basolateral surfaces (671).

Enterovirulent *E. coli*. (i) EIEC. EIEC infection in fully differentiated HT-29.cl19A, Caco-2, and T84 cells is followed by a fall in TER resistance, accompanied by rearrangements of TJ-associated ZO-1 and occludin, phosphorylation of occludin, and an increase in chloride secretion (672).

(ii) AIEC. Apical infection of fully differentiated T84 cell monolayers with the AIEC strain LF82 leads to a reduction in TER and increased paracellular flux accompanied by the redistribution of the TJ-associated ZO-1 protein (455) (Table 3). Interestingly, basolateral infection results in a more severe disruption of the epithelial barrier function (455). Infection of CEABAC10 transgenic mice expressing hCEACAMs with AIEC strain LF82, but not with nonpathogenic *E. coli*, leads to an increase in intestinal permeability and to the disruption of mucosal integrity in a type 1 pilus-dependent mechanism (673). The mechanism of the LF82-induced alteration in intestinal epithelial barrier remains to be investigated with the appropriate models of fully differentiated Caco-2 or T84 cell monolayers.

(iii) EPEC. tEPEC causes a decrease in TER of monolayers of fully differentiated Caco-2 and T84 cells (674–676) without affecting desmosomes (677) (Table 5). tEPEC infection of fully differentiated Caco-2 cells results in a decrease in the cell resting membrane potential (678). Activation of MLCK20 results in TER alteration in fully differentiated T84 cells infected with tEPEC (679). Intimate attachment of tEPEC involving the binding of intimin to Tir is necessary for alteration to occur at the TJs in fully differentiated Caco-2 and T84 cells (680, 681). In fully differentiated T84 cell monolayers, tEPEC-induced structural alterations in TJs, rearrangements in TJ-associated ZO-1, occludin, and claudin-1 proteins, and loss of TER all occur as a result the action of the T3SS translocated EspF effector (682). Intimate attachment of tEPEC to fully differentiated T84 cells disrupts the fence function of TJs and delocalizes basolateral proteins such as β1 integrin and Na^+/K^+ ATPase to the apical membrane (683). Intimate tEPEC attachment and the T3SS-translocated effectors EspF and Map are required for tEPEC to disrupt TJ integrity in fully differentiated Caco-2 cells and thus to increase the paracellular movement of molecules (684, 685). The T3SS-translocated effector protein NleA is involved in TJ disruption during tEPEC infection of fully differentiated Caco-2 cells as a result of the inhibition of COPII-dependent host cell protein trafficking (686, 687). In fully differentiated Caco-2 cells, infection by tEPEC induces a decrease of TER, a rearrangement in the distribution of TJ-associated ZO-1 and occludin, and the delocalization of occludin and flottilin-1 but not of claudin-1 and -3 from lipid rafts, thus allowing bacteria to penetrate the infected cells (688). tEPEC OMPs in fully differentiated Caco-2 cells monolayers induce changes in adherens junctions, leading to the dissociation of the cadherin/β-catenin complex and the cytoplasmic redistribution of β-catenins through the activation of PKC-α signaling (689). In fully differentiated Caco-2 cells, EspG and EspG2, through their activation of the host cysteine protease calpain, lead to a loss of TER and a rapid cell loss and necrosis, a phenomenon that is increased in the absence of Tir (690). The EspG effector and its homologue Orf3 are both involved in microtubule disruption in fully differentiated Caco-2 cells, but EspG alone has no effect on TER, whereas EspG combined with Orf3 decreases TER (691). tEPEC expresses two different types of EspG (EspGα and EspGβ) and EspG2, and these are responsible for microtubule disruption in fully differentiated Caco-2 cells (692), and EspG1 and its homologue EspG2 contributes to loss of barrier function via an undefined mechanism that may be linked to the disruption of the microtubule network (693). TJ organization in fully differentiated T84 cells is not affected by the tEPEC T3SS effector NleE activating Erk1/2 MAPK and NF-κB (694). The tEPEC EspF-induced increase in paracellular permeability and ZO-1 delocalization in fully differentiated Caco-2 cells does not relate to the tEPEC EspF-induced caspase 3, 8, and 9 cleavage (695, 696).

(iv) EHEC. EHEC in fully differentiated T84 cell monolayers decreases TER and increases the transepithelial passage of fluid by inducing reduced expression of TJ-associated ZO-1, occludin, and claudin-2, but not of claudin-1 or claudin-4 (697, 698), and without affecting desmosomes (677) (Table 5). In contrast to tEPEC, the EspF of which reduces TER, the EspF of EHEC does not have this effect on fully differentiated Caco-2 cell monolayers (685).

(v) EAEC. EAEC infecting fully differentiated T84 cell monolayers induces an AAF/II-dependent decrease in TER and the delocalization of the TJ-associated claudin-1 and, to a lesser degree, occludin (699) (Table 5).

(vi) Afa/Dr DAEC. Infection of fully differentiated Caco-2/TC7 cell monolayers by $Afa/Dr_{DAF/CEACAMs}$ DAEC expressing F1845 adhesin is followed by an increase in the paracellular permeability without any decrease in TER (700) (Table 5). The distribution of TJ-associated occludin and ZO-1 proteins is markedly altered,

whereas that of the zonula adherens-associated E-cadherin is not modified. These TJ lesions are independent of the recognition of hDAF by the F1845 adhesin, indicating that another Afa/$Dr_{DAF/CEACAMs}$ DAEC pathogenic factor is operant. Guignot et al. (701) have identified the secreted autotransporter toxin Sat, belonging to the subfamily of SPATE toxins (306, 506), as the virulence factor of Afa/$Dr_{DAF/CEACAMs}$ DAEC that promotes the TJ injuries in fully differentiated Caco-2/TC7 cell monolayers. Interestingly, these authors indicate that the *sat* gene is generally absent in Afa/$Dr_{DAF/CEACAMs}$ DAEC strains collected from the stools of children without diarrhea (16% positive) and is present in about half of the Afa/$Dr_{DAF/CEACAMs}$ DAEC strains collected from the stools of children with diarrhea.

Host Cellular Defense Responses

After recognition by PAMPs, PRRs rapidly trigger an array of antimicrobial immune responses through the induction of various inflammatory cytokines, chemokines, and type I interferon (IFN) (5, 6). In addition, opening of the TJs, coupled with the induced proinflammatory cell responses, leads to the paracellular passage of activated immune cells, including PMNLs and dendritic cells (DCs), from the lamina propria into the luminal compartment (165, 166, 179, 702).

Yersinia. Upregulation of proinflammatory cytokine IL-8 and monocyte chemotactic protein 1 (MCP-1), granulocyte-macrophage colony-stimulating factor (GM-CSF), and TNF-α genes and cytokine secretion have been reported in monolayers of confluent, undifferentiated Caco-2 cells which have been invaded by *Y. enterocolitica* (703) (Table 6). IL-8 is secreted by fully differentiated T84 cells in response to invasion by *Y. enterocolitica* via a mechanism dependent on YopB and YopD (704). *Yersinia*-induced IL-8 secretion has been investigated as a function of cell differentiation in T84 cells (314). IL-8 secretion occurs when *Y. enterocolitica* penetrates undifferentiated cells or fully differentiated cells via the basolateral domain by an *inv*-dependent mechanism. In contrast, when the apical domain of fully differentiated cells is infected, there is no IL-8 secretion and no invasion occurs. Infection of Caco-2 and T84 cells with *Y. enterocolitica* resulted in the activation of I-κB kinase alpha (IKKα) and IKKβ and increased NF-κB DNA binding activity (705).

In fully differentiated T84 cells, β1 integrin is strictly polarized to the basolateral membrane, and the transient microdiscontinuities resulting from the paracellular migration of PMNLs, mimicking an inflammatory situation, allows access to β1 integrin from the apical side of cultured cells (706). *Y. pseudotuberculosis* has been observed at sites where small discontinuities resulting from neutrophil transmigration are found (706). These observations indicate that the transient perturbations of monolayer continuity observed after the transepithelial migration of enterovirulent pathogen-induced PMNLs or inflammatory bowel diseases may be associated with a window of risk, during which enteroinvasive pathogens can gain access to the basolateral ligands necessary for unwanted cell entry.

Shigella. Upregulation of inducible nitric oxide synthase (iNOS) mRNA and nitric oxide (NO) production have been observed in undifferentiated Caco-2 cells infected with *S. flexneri* (707) (Table 6). Undifferentiated Caco-2 cells microinjected with *S. flexneri* bacterium-free supernatant or purified LPS lead to the translocation of NF-κB into the nucleus (708). In fully differentiated T84 cells, infection with *S. dysenteriae* results in the upregulation of proinflammatory cytokines IL-8, MCP-1, GM-CSF, and TNF-α (703). Like the T3SS-associated OspF, OspG, and IpaH effectors of *S. flexneri* that downregulate the host innate immune response, the OspB effector functions as a negative regulator, and in fully differentiated T84 cell it downregulates the *Shigella*-induced IL-8 secretion (709). Transcriptome analysis of undifferentiated Caco-2 cells infected by *S. flexneri* 5a has been conducted and compared to those of Caco-2 cells infected with a noninvasive *Shigella* mutant and of cells treated with TNF-α (710). The invasive and noninvasive strains both enhance the transcription of a common pattern of 240 genes, whereas in contrast, these genes are not induced by TNF-α treatment. Interestingly, induction of early genes and late genes has been observed, and among the set of 18 activated genes that mainly encode proinflammatory molecules, the invasive strain induces a dramatic increase in IL-8 gene transcription. *S. flexneri* infection of fully differentiated HT-29 Glc^- cells induces increased production of TNF-α mRNA, whereas no such increase developed in fully differentiated, mucin-secreting HT29-MTX cells, suggesting that mucus has a protective effect against the proinflammatory effect of *S. flexneri* (64). Nod1, which acts as a critical sensor of bacterial peptidoglycan fragments and in turn induces host cell innate immune responses (711), localizes at sites of *S. flexneri* entry in the plasma membrane of epithelial cells and is basolaterally localized in fully differentiated Caco-2/TC7 cells transfected with Nod1-HA (712).

Shigella attachment to the basolateral domain of fully differentiated T84 cells, triggering the transepithelial PMNL migration, has been found to be dependent on the presence of a *Shigella* 220-kb virulence plasmid (713). The T3SS-associated effectors OspC1, OspF, and OspZ play a role in the upstream induction of host signaling pathways, including the extracellular signal-regulated kinases 1 and 2 (Erk1/2), MAPKs required for PMNL transepithelial migration in *Shigella*-infected fully differentiated T84 cells (714, 715). In addition, *S. flexneri* LPS (716) and *Shigella* enterotoxin 2 (717) play a role in mediating IL-8 secretion and epithelium-derived signaling, which leads to the directed migration of PMLNs across the fully differentiated T84 cell monolayer.

In fully differentiated, mucin-secreting HT29-MTX cells, *S. dysenteriae* induces a cell response in which cross talk between IL-1β and Akt activated by the produced trefoil factor family peptide TFF3 inhibits the adherence of the pathogen and its invasion of cells (718).

Listeria. Upregulation of IL-8, MCP-1, GM-CSF, and TNF-α genes has been observed in fully differentiated T84 infection with *L. monocytogenes* (703) (Table 6). IFN-γ and IL-6 cause the overexpression of inducible nitric oxide synthase, thus reducing the intracellular growth of *L. monocytogenes* phagocytosed from the apical pole of fully differentiated Caco-2 cells (719). In addition, IL-6, but not IFN-γ, causes a partial restriction of *L. monocytogenes* in phagosomes and diminishes the number of bacteria residing within the cell cytoplasm. The role of intestinal P glycoprotein in host defenses against *L. monocytogenes* has been investigated using fully differentiated Caco-2 cells and a P-glycoprotein-overexpressing subclone, Caco-2/MDR (720). Overexpression of P glycoprotein in Caco-2/MDR cells leads to increased resistance to *L. monocytogenes* invasion, whereas P-glycoprotein inhibition leads to increased invasion. Several physiological and pathological processes, including host immune responses, are controlled by a family of very small, noncoding RNAs known as microRNAs that antagonize a number

TABLE 6 Overview of cellular responses in fully differentiated human colon cancer cell lines

Pathogen	Cell line	Virulence factor	Cell response	Reference(s)
Yersinia	Caco-2	Undetermined	Increase of IL-8, MCP-1, GM-CSF, and TNF-α mRNAs and secretion	703
	T84	YopB, YopD	IL-8 production	704
	T84	Undetermined	Differentiation-dependent basolateral IL-8 secretion	314
	T84	Undetermined	NF-κB activation	705
Shigella	Caco-2	Undetermined	Increase of IL-8, MCP-1, GM-CSF, and TNF-α mRNAs and secretion	703
	HT-29 Glc^-	Undetermined	TNF-α mRNA increase	64
	Caco-2	Undetermined	Activation of genes encoding proinflammatory molecules	710
	HT29-MTX	Undetermined	IL-1β- and Akt-dependent activation of trefoil factor family peptide TFF3	718
	T84	OspF, OspC1	Erk1/2-dependent PMNL transepithelial migration	715
	T84	OspZ	Erk1/2-dependent PMNL transepithelial migration	714
	T84	OspB, OspF	Downregulation of MAPK activation, PLMN transepithelial migration, and IL-8 secretion	709
	Caco-2/TC7	Undetermined	Basolateral localization of Nod1 at the bacterial entry site	712
Listeria	Caco-2	Undetermined	Increase of IL-8, MCP-1, GM-CSF, and TNF-α mRNAs and secretion	703
	Caco-2	Undetermined	IFN-γ and IL-6 production	719
	HT29-MTX	LLO	Stimulation of mucus exocytosis, upregulation of genes coding for secreted and membrane-bound mucins	62, 63, 79, 80
	Caco-2	LLO	Alteration of miR-146b, miR-16, let-7a1, miR-145, and miR-155 microRNAs	721
Campylobacter	Caco-2	Undetermined	Activation of Erk1/2, p38, and JNK	657, 722
	Caco-2	Undetermined	Activation of NF-κB and AP-1	657
	Caco-2 and T84	Undetermined	IL-8 production	657, 723
	T84	OMVs	Lipid raft-dependent IL-8 production	724
	T84	Flagella	Activation of NF-κB and TLR5	725
	T84	Flagella and CDT	TLR4-dependent NF-κB signaling controlling secretion of IL-8 and TNF-α	726
	Caco-2 and T84	LOS	Production of CXCL10	348
	T84	Undetermined	CDT-independent induction of oncosis	728
	Caco-2	Undetermined	Increased hBD-2 and hBD-3 gene expression	723, 729
Salmonella	Caco-2	Undetermined	Activation of Erk1/2 and JNK	730
	Caco-2 and T84	Undetermined	Activation of NF-κB	705
	Caco-2	Undetermined	Increase of IL-8, MCP-1, GM-CSF, TNF-α, CXCL1 (GRO-α), CXCL3 (GRO-γ), and RANTES mRNAs	703, 732
	T84	Undetermined	PI3K/Akt pathway reduces Erk1/2 activation and IL-8 production	733
	Caco-2 and T84	Undetermined	Upregulation of iNOS and COX-2 regulating chloride secretion	672
	Caco-2	Flagellin	Induction of I-κBα degradation, NF-κB nuclear translocation, and iNOS	736
	T84	SopE2 and flagellin	Cooperation for activation of MAPKs and IL-8 production	737
	T84	TviA regulator	By reducing flagellin secretion in turn reduces IL-8 production	738
	T84	Undetermined	PMNL transepithelial migration	739, 740, 742
	T84	Chemotactic factor	PMNL transepithelial migration	739, 741
	T84	IL-8, hepoxilin A3 (HXA3)	Cooperation for PMNL epithelial transmigration	741, 743, 744
	T84	SipA	Activation of ezrin regulating apical transporters NHE-3, CFTR, and MRP2, in turn increasing apical expression of MRP2 controlling HXA3 release for PMNL transepithelial migration	743
	T84	SipA	Induces PKC-α-dependent PMNL transepithelial migration	747
	HT29-MTX	Undetermined	Secretion-dependent production of mucus preventing infection	748, 749
	Caco-2	Flagella	Increase of hBD-2 expression	750-753
Vibrio cholerae	T84	Undetermined	IL-8 secretion	756
	T84	Flagellin	MAPK- and NF-κB-dependent IL-8 production involving TLR5	758
	T84	Undetermined	Increase of upregulation of IL-1α, IL-1β, TNF-α, IL-8, and MCP-1 mRNAs	759
	T84	Protease PrtV and hemolytic exotoxin VCC	Production of IL-8 and TNF-α	760
	Caco-2	Hemolytic exotoxin VCC	Induction of autophagy	761
ETEC	Caco-2	LT	Stimulation of cAMP adenylate cyclase in fully differentiated Caco-2 cells	568–572
	T84	ST	Stimulation of cGMP adenylate cyclase	575–577
EIEC	Caco-2, T84	Undetermined	Activation of NF-κB	705

(Continued on following page)

TABLE 6 (Continued)

Pathogen	Cell line	Virulence factor	Cell response	Reference(s)
AIEC	T84	Flagella	*fimA*-dependent basolateral IL-8 secretion	454
	Caco-2BBe, T84		Secretion of IL-8 and CCL20	449
	Caco-2BBe, T84		Transepithelial migration of DCs and PMNLs	449
	Caco-2BBe	Undetermined	Suppression of STAT1 signal transduction	767
	Caco-2	Undetermined	Expression of Nod1 protein	768
tEPEC	T84	Undetermined	NF-κB-dependent IL-8 production	769
	Caco-2	NleE	Erk1/2- and NF-κB-dependent PMNL transepithelial migration	694, 714
	T84	Flagellin FliC	Erk1/2-, p38-, and JNK-dependent IL-8 secretion	770, 771
	T84	Undetermined	MAPK- and NF-κB-dependent activation of IL-8, MCP-1, MIP-3α, and hBD-2 genes	772
	Caco-2, T84	Undetermined		773, 774
	Caco-2	BFP, flagella, EspA, EspC	NF-κB- and PKC-ζ-dependent IL-8 production	776
	Caco-2, T84	T3SS effectors	IL-8 and CCL20 production	771, 772
	Caco-2	NleE, NleH1, NleC	Suppressed MAPK- and NF-κB dependent IL-8 production	777
	Caco-2	Undetermined	Inhibition of NF-κB leads to inhibition of iNOS expression	778
	Caco-2	OMPs	MAPK- and NF-κB-dependent upregulation of iNOS	779
	Caco-2	EspF	Activation of caspases 3, 8, and 9 cleavage	695, 696
aEPEC	Caco-2	Flagella	Early but not late IL-8 production in fully differentiated Caco-2 cells	488, 782
	HT29-MTX	Undetermined	Mucus hypersecretion (whereas not for tEPEC)	783
EHEC	Caco-2	Undetermined	Erk1/2-, p38-, NF-κB, and AP-1-dependent increase of mRNA expression and IL-8 production	784, 785
	Caco-2	Flagellin	MAPK and NF-κB activation for IL-8 secretion	784, 786
	Caco-2	Stx1, Stx2	Increase of IL-8 mRNA	793
	T84	HCP	Erk1/2-, p38-, and JNK-dependent IL-8 and TNF-α but not IL-2, IL-6, or IL-10 production	787
	Caco-2, T84	Stx1	Downregulation of IFN-γ-induced iNOS mRNA expression and NO production heme oxygenase-1	208, 788, 791
	Caco-2	OmpA	IL-1, IL-10, and IL-12 production and DC transepithelial migration	789
	T84	Stx	Inhibition of PI3K/Akt-, NF-κB-dependent CCL20 and IL-8 gene transcription and chemokine production	792
EAEC	Caco-2	Flagellin	p38- and TLR5-dependent IL-8 production	794, 796, 797
	T84	AAF	Upregulation of genes encoding IL-8, IL-6, TNF-α, CXCL1, CXCL3, ICAM-), GM-CSF, and IL-1α	799
	T84	Undetermined	PMNL transepithelial migration involving 12/15-LOX pathway and arachidonic acid-derived lipid PMNL chemoattractant	800
	T84	Undetermined	Signs of cell death	507
Afa/$Dr_{DAF/CEACAMs}$ DAEC	Caco-2	Dr, F1845	Recognition of brush border hDAF leads to Erk1/2-, p38-, and JNK-dependent basal production of IL-8 and PMNL transepithelial migration	807
	Caco-2/TC7	Dr, F1845		
	T84	Flagella or not	PMNL transepithelial migration leads to synthesis of TNF-α and IL-1β, in turn promoting hDAF upregulation at brush border increasing bacterial adhesion, and abnormal hDAF basolateral expression	808
	Caco-2, T84	Flagella	TLR5-dependent IL-8 production	801, 802, 803
	Caco-2/TC7	Dr	hDAF- and Erk1/2-dependent IL-8 production through Dr adhesin released by norepinephrine affecting the induction of gene *draC* encoding the usher	804, 805
	T84	F1845	Induction of hypoxia-induced factor 1α (HIF-1α) for production of IL-8 and VEGF	821
	Caco-2	Afa-III	Upregulation of the inflammation-associated molecule MICA mediated by interaction with hDAF	818
	Caco-2	Dr, F1845	Antibacterial cell response leads to destruction of adhering bacteria at the brush border	54
	T84	F1845	Production of bioactive VEGF through hDAF recognition and subsequent Src protein kinase activation upstream of the activation of the Erk1/2 and Akt signaling pathways	820
aDAEC	T84	Undetermined	Production of IL-8	806

of target mRNAs. Whether *Listeria monocytogenes* or listeriolysin O (LLO) alters the expression of microRNAs has been investigated using Caco-2 cells (721). Five microRNAs (miR-146b, miR-16, let-7a1, miR-145, and miR-155) are significantly deregulated following *L. monocytogenes* infection. Interestingly, during infection with wild-type bacteria or LLO-deficient bacteria or after treatment with purified LLO, miR-155, which plays an important role in inflammatory responses, is induced. Moreover,

miR-155 is downregulated following infection with an internalin mutant.

L. monocytogenes stimulates mucus exocytosis in fully differentiated mucin-secreting HT29-MTX cells through the action of LLO (79, 80). The *MUC3*, *MUC4*, and *MUC12* genes, which encode membrane-bound mucins, are upregulated in LLO-stimulated HT29-MTX cells, and the secretion of the gel-forming MUC5AC mucin develops without upregulation of the *MUC5AC* gene (62). Increased expression of membrane-bound MCU4 and MUC12 at the brush border results in inhibition of the cell entry of *L. monocytogenes* into the HT29-MTX cells (63).

***C. jejuni*.** Infection of fully differentiated Caco-2 cell monolayers or human colonic explants results from an increase in proinflammatory MAPKs Erk1/2, p38, and c-Jun N-terminal kinase (JNK) (722) (Table 6). Apical infection of fully differentiated T84 cells with *C. jejuni* causes activation of the transcription of the transcription factors NF-κB and AP-1, the phosphorylation of the Erk1/2, p38, and JNK MAPKs, and the basolateral secretion of IL-8 (657). It has been noted that basolateral infection with *C. jejuni* causes greater secretion of IL-8 than apical infection (657). In fully differentiated Caco-2 and T84 cells, *C. jejuni* induces strong IL-8 secretion (723). *C. jejuni*-produced OMVs containing a large set of bacterial proteins, including CDT, exert a cytotoxic activity and induce lipid raft-dependent IL-8 production in fully differentiated T84 cells (724). Infection of fully differentiated T84 cells with *C. jejuni* upregulated DCs and T-cell chemokine gene transcription and secretion through the activation of NF-κB signaling, but this is independent of TLR5 activation by flagellin (725). *C. jejuni*, by use of its flagellum and cytolethal distending toxin, increases the secretion of both IL-8 and TNF-α via TLR4-dependent NF-κB signaling (726). *C. jejuni* isolates expressing ganglioside-like LOS increase the secretion of the T-cell attractant CXCL10 in fully differentiated Caco-2 and T84 cells (348). *C. jejuni* infection in fully differentiated T84 cell monolayers disrupts epithelial TLR9 signaling, thus exerting a protective effect (727). Fully differentiated T84 cells when invaded by *C. jejuni* display oncosis that is independent of cytolethal distending toxin activity (728).

In undifferentiated Caco-2 cells, *C. jejuni* increases hBD-2 and hBD-3 gene expression without modifying the expression of the hBD-1 gene (723, 729). This is a host cell defense response, since the recombinant human β-defensins hBD-2 and hBD-3 exhibit bactericidal activity against *C. jejuni* that is characterized by bacterial cell membrane damage in the case of hBD-3 (729).

***Salmonella* spp.** *S.* Typhimurium induces the activation of the proinflammatory signaling Erk1/2 and JNK in fully differentiated Caco-2 cells (730) (Table 6). The upregulated transcription and expression of downstream targets genes of NF-κB, which are not necessary for the cell entry of *S. enterica* serovar Dublin and *S.* Typhimurium (731), are key components in the inflammatory response to *S.* Dublin infecting undifferentiated Caco-2 cells and fully differentiated T84 cells (705). *S.* Dublin infection of undifferentiated Caco-2 cells results in the upregulation of proinflammatory IL-8, MCP-1, GM-CSF, TNF-α, CXCL1 (GRO-α), CXCL3 (GRO-γ), and RANTES (regulated and normal T-cell expressed and secreted) genes (703, 732). *Salmonella* activates the PI3K/Akt pathway in fully differentiated T84 cells, resulting in altered activation of Erk1/2, which in turn attenuates IL-8 production (733). The fully differentiated cell line T84 infected with *S.* Typhimurium secretes IL-6, a proinflammatory cytokine involved in neutrophil degranulation and lymphocyte differentiation (734). *S.* Typhimurium-induced IL-6 production in undifferentiated Caco-2 cells develops via the Erk1/2 and NF-κB signaling pathways but not via the p38 MAPK, JNK, or PI3K/Akt signaling pathway (735). The upregulation of iNOS and cyclooxygenase 2 (COX-2) by *S.* Dublin in fully differentiated Caco-2 and T84 cells can in turn modulate chloride secretion and barrier function in intestinal epithelial cells (672). Flagellin, the primary component of bacterial flagella, is a potent activator of TLR5 signaling localized at the basal domain of enterocytes and is a major proinflammatory determinant of *Salmonella*. The flagellum of *S.* Dublin induces I-κBα degradation, the nuclear translocation of NF-κB, and an increase of iNOS (736). SopE2, a *Salmonella* guanine nucleotide exchange factor, cooperates with flagellin to activate MAPK and IL-8 production (737). *S. enterica* serovar Typhi, which does not elicit neutrophil infiltrates in the human intestinal mucosa, expresses the B locus which is involved in reducing IL-8 production in fully differentiated T84 cells, because the TviA regulatory protein reduces flagellin secretion and, in turn, reduces IL-8 production (738).

McCormick et al. (739) were the first to report that after *S.* Typhimurium apical infection of a fully differentiated T84 cell monolayer, PMNLs subsequently placed on the basolateral domain of the monolayers start transepithelial migration without compromising the monolayer integrity as assessed by TER and measurements of ion transport. Investigation of the potentials of various *Salmonella* serovars, including *S.* Typhimurium, *S.* Enteritidis, *S.* Typhi, *S. enterica* serovar Paratyphi, *S. enterica* serovar Pullorum, and *S. enterica* serovar Arizonae, to induce the transepithelial migration of PMNLs has revealed that strains or serovars that induce diffuse enteritis in human beings do induce transmigration, whereas those that do not induce diffuse enteritis in human beings do not (740). In contrast, the ability to enter fully differentiated T84 cells does not differentiate between strains or serovars that induce diffuse enteritis and those which do not (740). The *Salmonella*-induced transepithelial migration of PMNLs is not attributable to the classical pathway by which an enteropathogenic bacterium induces the migration of PMNLs, because even though the infected cells release the potent PMNL-chemotactic IL-8, this cytokine is not only responsible for the *Salmonella*-induced transepithelial transmigration of PMNLs, suggesting the involvement of a chemotactic factor(s) (739, 741). Analyzing the phenomenon, McCormick et al. (742) provided evidence that the primary role for basolateral secretion of IL-8 by the fully differentiated T84 cells is the recruitment of PMNLs through the matrix to the subepithelial space, rather than directing the final movement of PMNLs across the epithelium. In fully differentiated T84 cell monolayers, *S.* Typhimurium recruits PMNLs and induces their transepithelial migration by the coordinated production of two potent PMNL chemoattractants, the basolateral IL-8 and the apical hepoxilin A3 an eicosanoid lipid (741, 743, 744). The *S.* Typhimurium effector protein SipA has been found to be both necessary and sufficient for the epithelial transmigration of PMNLs in fully differentiated T84 monolayers (745). In particular, SipA facilitates the apical release of HXA3 via an increase in the protein expression of the ATP-binding cassette transporter multidrug resistance-associated protein 2 (MRP2) (743). Mechanistically, SipA activates ezrin, a member of the ERM protein family that regulates the localization and functionality of intestinal apical transporters, including NHE-3, and CFTR and

MRP2 in polarized epithelial cells, in turn increasing the apical expression of MRP2, which controls the release of HXA3 and hence the induction of the transepithelial migration of PMNLs (746). In addition, SipA, by initiating an ADP-ribosylation factor-6- and phospholipase D-dependent lipid-signaling cascade, directs the specific activation of PKC-α and subsequent PMNL migration (747).

The mucus layer present at the apical domain of fully differentiated, mucin-secreting HT29-MTX cells creates a barrier against *S.* Typhimurium cell invasion (748). Chloride secretion induced in fully differentiated, mucin-secreting HT29-MTX cells results in the shedding of the mucus gel covering the apical cell domain and a reduction in mucus gel density and barrier properties, which in turn limits the innate defense mechanism against *S.* Typhimurium invasion (749).

hBD-2, but not hBD-1, is upregulated after *S.* Enteritidis, *S.* Typhimurium, *S.* Typhi, or *S.* Dublin infection of undifferentiated Caco-2 cells (750, 751) through the action of the filament structural FliC protein, which, after binding to membrane-associated gangliosides (752), increases the binding of NF-κB to hBD-2 gene promoter sequences (750, 753). The secretory leukocyte proteinase inhibitor expressed by fully differentiated Caco2-BBe clone cells, T84 cells, and the HT29-Cl.19A cell subpopulation in proinflammatory situations exerts an antibacterial activity against *S.* Typhimurium (754). The number of viable, internalized *S.* Typhimurium bacteria in undifferentiated Caco2 cells stably transfected with the CARD15/NOD2 expression plasmid is lower than that in untransfected Caco2 cells or Caco2 cells transfected with a mock transfectant (755).

V. cholerae. *V. cholerae* is thought to be a prototypical noninflammatory enteric pathogen, although innate immune responses such as the production of proinflammatory cytokines and expression of bactericidal proteins have been reported (401) Moreover, how the adaptive immune response to cholera mediates protection against subsequent disease is unknown. *V. cholerae* induces the production of IL-8 in fully differentiated T84 cells (756) (Table 6). Purified flagella and secreted flagellin proteins, FlaC and FlaD, of *V. cholerae* induce IL-8 production via TLR5 in undifferentiated parental HT-29 cells (757). The flagellin-induced TLR5-dependent IL-8 production in fully differentiated T84 cells develops through the activation of MAPKs and NF-κB (758). Upregulation of IL-1α, IL-1β, TNF-α, IL-8, and MCP-1 has been found in *V. cholerae*-infected fully differentiated T84 cells, whereas a striking dissimilarity in cytokine expression was observed in infected fully differentiated Caco-2 cells (759). The increased paracellular permeability and production of IL-8 and TNF-α in *V. cholerae*-infected fully differentiated T84 cells have been found to be triggered by the *V. cholerae* protease PrtV and *V. cholerae* cytolysin (VCC) (760).

The appearance of large intracellular vacuoles displaying the hallmarks of autophagosomes and the induced autophagy in epithelial cells, including undifferentiated Caco-2 cells, intoxicated with the hemolytic exotoxin known as VCC are cell survival responses, since inhibiting autophagy reduces the survival of VVC-intoxicated cells (761). Cholix toxin, a *V. cholerae* ADP-ribosylating cytotoxin, which utilizes eukaryotic elongation factor 2 as a substrate, promotes caspase-dependent apoptosis in HeLa cells but not in Caco-2 cells (762).

CT transcriptionally downregulates the expression of AMPs LL-37 and HBD-1 in fully differentiated Caco-2 cells by activating protein kinase A, Erk1/2 MAPK, and Cox-2 downstream of cAMP accumulation (574).

The chitin-binding protein GbpA of *V. cholerae* has been found to be responsible for NF-κB-dependent upregulation of the *MUC2*, *MUC3*, and *MUC5AC* genes in a mucus-secreting HT-29 cell line (763).

Enterovirulent *E. coli*. (i) EIEC. Infection of Caco-2 and T84 cells with EIEC results in the activation of IKKα and IKKβ and increases NF-κB DNA-binding activity (705) (Table 6). Infection of undifferentiated Caco-2 cells and of fully differentiated T84 cells with EIEC results in the coordinate expression and upregulation of IL-8, MCP-1, GM-CSF, and TNF-α (703) and in the upregulation of iNOS mRNA and protein expression and NO production (707). IKKβ and NF-κB activation was observed in undifferentiated Caco-2 cells in response to EIEC infection (764). The EIEC-induced expression of iNOS, and consequently of COX-2, is accompanied by increased basal and stimulated chloride secretion (672, 765). Reduction of endogenous MUC17 in undifferentiated Caco-2 cells is associated with enhanced bacterial invasion in response to exposure to EIEC infection (765). Moreno et al. (766) compared the innate and adaptive immune responses to EIEC and *S. flexneri* infection. EIEC triggered DC activation to produce IL-10, IL-12, and TNF-α, whereas *S. flexneri* induced only the production of TNF-α. Unlike *S. flexneri*, EIEC markedly increased the expression of TLR4 and TLR5 in DCs and diminished the expression of costimulatory molecules that may cooperate to inhibit $CD4^+$ T-lymphocyte proliferation.

(ii) AIEC. AIEC strains induce a *fimA*-dependent basolateral IL-8 secretion in fully differentiated T84 cells (454). A flagellum-dependent AIEC O83:H1-induced basolateral secretion of IL-8 and CCL20 has been observed in fully differentiated Caco-2BBe clone cells and T84 cells (449). Moreover, AIEC strains O83:H1 and LF82 both stimulate the transepithelial migration of DCs and PMNLs in fully differentiated Caco-2BBe clone cells and T84 cells (449). Infection of fully differentiated Caco-2BBe clone cells with EIEC strains LF82, O83, and UM146 results in the suppression of STAT1 signal transduction, suggesting a novel mechanism by which EIEC evades host immune responses to the infection (767).

The nucleotide-binding oligomerization domain containing 1 gene encodes the pathogen-sensing NOD1 pattern recognition receptor, leading to downstream responses characteristic of innate immunity. Nod1 protein has been found to be produced in fully differentiated Caco-2 cells after invasion by AIEC strain LF82, suggesting that the infected cell engages a transcriptional activation of the gene (768).

(iii) EPEC. Activation of NF-κB by tEPEC in fully differentiated T84 cells leads in turn to IL-8 transcription (769) (Table 6). The tEPEC flagellin FliC promotes the secretion of IL-8 from fully differentiated T84 cells by activating Erk1/2, p38, and JNK MAPKs (770). tEPEC flagellin triggers the secretion of IL-8 from fully differentiated Caco-2 and T84 cells, to a greater extent as a result of basolateral infection than as a result of apical infection (771). In fully differentiated Caco-2 cells, the flagella of tEPEC induce both early and late activation of IL-8, MCP-1, macrophage inflammatory protein 3α (MIP-3α), and hBD-2, accompanied by MAPK and NF-κB activation (772). PKC is activated in tEPEC-infected fully differentiated T84 cells (773), and the tEPEC-induced NF-κB activation that upregulates IL-8 expression in fully differentiated Caco-2 cells develops as a result of PKC-ζ signaling activation (774). Epidermal growth factor receptor plays a role in tEPEC-

induced Erk1/2 MAPK activation and IL-8 production in fully differentiated Caco-2 cells (775). A combination of bacterial agonists, including flagella, LPS, BFP, EspA, and EspC, has been found to be necessary for the production of the IL-8 and CCL20 proteins in fully differentiated Caco-2 cells (776). Following infection of Caco-2 cells, but not of T84 cells, IL-8 secretion and MAPK activation are inhibited by a mechanism dependent on the delivery of T3SS effectors, suggesting that some translocated bacterial effectors suppress inflammatory responses (771). The T3SS effectors NleE (non-locus, LEE-encoded effector), NleH1, and NleC prevent NF-κB nuclear translocation and suppress p38 MAPK activation, thus suppressing IL-8 release in fully differentiated Caco-2 cells (777). tEPEC inhibits iNOS expression at the transcriptional level, by both direct and indirect mechanisms, and also at posttranscriptional levels, several of which are related to the inhibition of NF-κB (778). In contrast, the tEPEC OMPs upregulate iNOS, induce nitrite production, and activate NF-κB and MAPKs in fully differentiated Caco-2 cells (779). Moreover, tEPEC induces caspase-dependent host cell death in fully differentiated Caco-2 and T84 cells (695, 780).

In fully differentiated T84 cells, tEPEC triggers an *eaeB*-dependent transepithelial migration of PMNLs (781). Without affecting TJ organization, the transepithelial migration of PMNLs across fully differentiated T84 cells (694) is promoted by the tEPEC T3SS effector NleE activating Erk1/2 MAPK and NF-κB (714).

The flagellum of aEPEC plays a role in adhesion and in the aEPEC-induced early, but not late, IL-8 production in fully differentiated Caco-2 cells (488, 782). aEPEC adhering to fully differentiated, mucin-secreting HT29-MTX cells induces mucus hypersecretion, whereas tEPEC does not (783). The mucins produced are the secreted mucins MUC2 and MUC5AC and the membrane-bound mucins MUC3 and MUC4. The transcription of the MUC5AC and MUC4 genes was transiently upregulated after aEPEC infection. aEPEC exploits the membrane-bound mucins for its growth, whereas tEPEC does not.

(iv) EHEC. Infection of fully differentiated Caco-2 cells with EHEC activated p38 and Erk1/2 MAPKs and induced the nuclear translocation of NF-κB and AP-1-binding activity for increasing IL-8 mRNA and the production of the protein (784, 785) (Table 6). The MAPK and NF-κB pathways leading to IL-8 secretion are also activated by isolated EHEC H7 flagellin added to either the apical or basolateral surface of fully differentiated Caco-2 cells (784). The EHEC H7 flagellin-induced production of IL-8 in fully differentiated Caco-2 cells develops together with the phosphorylation of the epidermal growth factor receptor (786). Moreover, EHEC expressing HCP induces significant release of IL-8 and TNF-α, but not of IL-2, IL-6, or IL-10, in fully differentiated T84 cells via Erk1/2, p38, and JNK MAPK signaling activation (787). tEHEC is able to downregulate IFN-γ-induced iNOS mRNA expression and NO production in fully differentiated Caco-2 and T84 cells by suppressing the STAT-1-dependent transcription of the gene encoding iNOS following activation of the enzyme heme oxygenase 1 (208, 788). In addition, it has been observed that the adhesive factor of EHEC OmpA induces IL-1, IL-10, and IL-12 cytokine production by DCs and the DC transepithelial migration across fully differentiated Caco-2 cell monolayers (789). In fully differentiated Caco-2 cells infected with EHEC, the observed tEHEC-induced downregulation of IFN-γ-induced iNOS mRNA expression and NO production (208, 788, 790) results from the action of its Stx in inhibiting the IFN-γ-mediated proinflammatory pathway by decreasing Stat-1 tyrosine phosphorylation (791). EHEC Stx also functions as a modulator of the innate immune response of human enterocytes, since Stx inhibits the NF-κB-dependent CCL20 and IL-8 gene transcription and chemokine production via a PI3K/Akt-dependent signaling pathway in fully differentiated T84 cells (792). Other cell responses and deleterious cellular effects resulting from the action of Stxs on STEC have been observed in fully differentiated intestinal cells. Proinflammatory cytokine mRNAs, including IL-8 mRNA, are induced by Stx1 and Stx2 in fully differentiated Caco-2 cells (793).

(v) EAEC. EAEC strains have been shown to cause the release of IL-8 from fully differentiated Caco-2 cells (794), an effect mediated by the flagella and flagellin protein (795) and involving p38 MAPK signaling via activation of the TLR5 receptor located in the basolateral domain of the cells (796, 797) (Table 6). In addition, plasmid-encoded factors may play a role in EAEC IL-8 induction (798). In EAEC-infected fully differentiated T84 cells, the upregulation of genes encoding IL-8, IL-6, TNF-α, CXCL1, CXCL3, intercellular adhesion molecule 1 (ICAM-1), GM-CSF, and IL-1α has been observed, as a result of the presence of AAF adhesin (799). In addition, two proinflammatory effects triggered by EAEC flagella or AAF adhesin have been shown to be induced in undifferentiated Caco-2 cells (776).

The migration of PMNLs across fully differentiated T84 cell monolayers infected with EAEC strain 042 is mediated by a host cell signaling cascade involving the 12/15-LOX pathway and leads to apical secretion of an arachidonic acid-derived lipid PMN chemoattractant (800). Moreover, the transepithelial migration of PLMNs in turn promotes the enhanced attachment of EAEC 042 to T84 cells (800).

(vi) Afa/Dr DAEC. Flagellated and nonflagellated Afa/$Dr_{DAF/CEACAMs}$ DAEC strains were able to induce the production of IL-8 in fully differentiated Caco-2 and T84 cells, and flagella isolated from flagellated DAEC recognize the basolateral TLR5 and induce the production of IL-8 (801, 802) (Table 6). In fully differentiated Caco-2 cells, motile Afa-positive DAEC strains isolated from patients with diarrhea induce a greater TLR5-dependent IL-8 secretion than strains isolated from healthy individuals (803). To explain the phenomenon, the authors speculated that an additional virulence factor other than Afa adhesins and motility causes the loosening of TJs that allows flagellin to reach TLR5 located on the basolateral side of the epithelium. It may be possible that the phenomenon results from the action of the Sat toxin opening the TJs in fully differentiated Caco-2/TC7 cells (701). Afa/$Dr_{DAF/CEACAMs}$ DAEC strains expressing Dr adhesin are able to induce cell signaling and a proinflammatory response at a distance from bacteria adhering to the intestinal brush border. Indeed, NE-induced release of Dr fimbriae from Afa/$Dr_{DAF/CEACAMs}$ DAEC has been observed. This is due to the differential induction of the genes *draC*, which encodes the usher, and *draE*, which encodes the major fimbrial subunit (804). Like for Afa/$Dr_{DAF/CEACAMs}$ DAEC apically infecting intestinal cells, the released Dr fimbriae are able to induce hDAF-dependent phosphorylation of Erk1/2 MAPK and the production of IL-8 in fully differentiated Caco-2/TC7 cells (805). It is noteworthy that non-Afa/Dr DAEC strains are also able to induce IL-8 production in fully differentiated T84 cells (806).

By use of their Dr or F1845 adhesins, after recognizing the brush border hDAF, Afa/$Dr_{DAF/CEACAMs}$ DAEC strains promote the epithelial transmigration of PMNLs across fully differentiated T84 monolayers accompanied by the basolateral secretion of IL-8

through a mechanism involving activation of Erk1/2, p38, and JNK MAPKs (807). F1845-induced transepithelial migration of PMNLs triggers the synthesis of TNF-α and IL-1β, which in turn promotes the upregulation of brush border-associated hDAF, increasing the adhesion of Afa/$Dr_{DAF/CEACAMs}$ DAEC to the brush border, and abnormal expression of hDAF at the basolateral domain of the fully differentiated T84 cells (808). PMNLs that have transmigrated across fully differentiated T84 monolayers show an elevated global caspase activity, indicative of apoptosis, and a reduced capacity to phagocytose Afa/$Dr_{DAF/CEACAMs}$ DAEC (809). Neutrophil extracellular traps (NETs) are composed of a nuclear DNA backbone associated with antimicrobial peptides, histones, and proteases (810–812). Wild-type Afa/$Dr_{DAF/CEACAMs}$ DAEC strain C1845 infecting the human myeloid cell line PLB-985, which differentiates into fully mature neutrophils, promotes the projection of NETs which entrap and kill bacteria (813). This PMNL response has a cytotoxic consequence for intestinal cells. Indeed, the induced NETs, by contact with fully differentiated Caco-2/TC7 cells, promote a dramatic disruption of the brush border F-actin cytoskeleton, which can be considered a new Afa/Dr $DAEC_{DAF/CEACAMs}$ cell-deleterious effect occurring in an inflammatory situation (813). Upregulation of the inflammation-associated molecule MICA, a distant homologue of major histocompatibility complex (MHC) class I molecules expressed in the normal intestinal epithelium (814) which acts as a ligand of the NKG2D-activating receptor (815–817), has been found in fully differentiated Caco-2 cells infected by Afa/$Dr_{DAF/CEACAMs}$ DAEC strains expressing AfaE-III adhesin, an effect mediated by the specific interaction between the bacterial adhesin and hDAF (818). Higher levels of MICA have been found on the surface of epithelial cells in colonic biopsy specimens from Crohn's disease patients than in those from controls (819).

Adhesion of Afa/$Dr_{DAF/CEACAMs}$ DAEC expressing F1845 adhesin at the brush border of fully differentiated Caco-2 cells triggers an antibacterial cell response, since at a late time of adhesion a dramatic bacterial cell alteration occurs in the adhering bacteria, indicating an epithelial cell-induced bacterial lysis (54), a phenomenon that correlates with the cell differentiation-dependent expression of AMPs by Caco-2 cells (54, 143).

Afa/$Dr_{DAF/CEACAMs}$ DAEC bearing the F1845 adhesin induces the production of bioactive vascular endothelial growth factor (VEGF) in fully differentiated T84 cells as a result of the recognition of hDAF and subsequent Src protein kinase activation upstream of the activation of the Erk1/2 and Akt signaling pathways (820). The Afa/Dr $DAEC_{DAF/CEACAMs}$ expressing F1845 adhesin induces the upregulation of hypoxia-induced factor 1α (HIF-1α), which triggers the production of IL-8 and VEGF in fully differentiated T84 cells. Concomitantly, infected fully differentiated T84 cells display a loss of E-cadherin and cytokeratin 18 and a rise in fibronectin, indicating that the bacteria may induce an epithelial-to-mesenchymal transition-like phenotype (821). This suggests that Afa/$Dr_{DAF/CEACAMs}$ DAEC could play a role in angiogenesis, inflammation, and some aspects of intestinal cancer initiation (822). The same HIF-1α upregulation and activation of VEGF/VEGF receptor (VEGFR) signaling has been observed in fully differentiated T84 cells infected with the AIEC strain LF82 (823).

Interaction with Caco-2-Derived M-Like Cells

In the case of enteroinvasive pathogens, experimental data indicate that at least during the early stages of infection, the M cells of the follicle-associated epithelium transport the pathogens (221, 222). In the coculture model of fully differentiated Caco-2 cells and murine lymphocytes from Peyer's patches, *Y. enterocolitica* does not adhere to the apical membrane of fully differentiated cells but does adhere to and is internalized by M-like cells expressing β1 integrins at their apical surface, which are recognized by the invasin of *Yersinia* (824). After internalization, the bacteria are localized within LAMP-1-negative vacuoles. Similar binding of *Y. enterocolitica* to M-like cells, but not to fully differentiated, brush border-expressing cells, has been observed using the coculture system of clones of fully differentiated Caco-2 cl1 cells and freshly isolated human blood lymphocytes (227).

At least at the early stage of infection, M cells of the follicle-associated epithelium transport *Shigella* (222). Surprisingly, despite the establishment of coculture system involving fully differentiated Caco-2 cells and freshly isolated BALB/c mouse Peyer's patch lymphocytes or the human lymphoblast-like Raji B cell line mimicking M cells, there has been no published description of the interaction between *S. flexneri* and the M cell-like cells.

Using the coculture model of parental fully differentiated Caco-2 cells and freshly isolated mouse Peyer's patch lymphocytes that mimics the follicle-associated epithelium, Daniels et al. (825) concluded that *L. monocytogenes* does not require or specifically use M cells to cross the gut. In contrast, in an coculture model composed of fully differentiated Caco-2BBe 1 clone cells with murine Peyer's patch lymphocytes, transcytosis of *L. monocytogenes* production has been observed independently of bacterial hemolysin and internalin (826).

In addition, *V. cholerae* binding and penetration, the presence of bacteria in an intraepithelial pocket containing a lymphocyte, and bacterial translocation from human M-like cells have been visualized using the coculture model of M-like cells composed of isolated BALB/c mouse PP lymphocytes and parental fully differentiated Caco-2 cells or Caco-2 cl1 clone cells (223, 224). The participation of the secretory IgA receptor colocalizing with the CT receptor ganglioside GM1 in the uptake of *V. cholerae* has been demonstrated using the coculture model of M-like cells composed of Raji cells, human B lymphocytes, and parental, fully differentiated Caco-2 cells (559, 827). CT remains in the proximity of the bacteria as they are trafficked through the M-like cells (559). Moreover, *V. cholerae* binding and transcytosis in M-like cells have been found to depend on bacterial viability, since heat treatment of the bacteria causes a loss of binding to GM1 that is correlated with a significant decrease in uptake and transcytosis (559). Importantly, it has been noted that *V. cholerae* bacteria are found only attached to the brush border of fully differentiated Caco-2 cells (223, 224), and intracellular *V. cholerae* has never been observed in fully differentiated cells, whether they are adjacent to M-like cells or not (224, 559).

Salmonella translocation has been observed in the coculture system of parental Caco-2 cells and the human lymphoblast-like Raji B cell line mimicking M cells (828) independently of factors encoded by T3SS-1 and T3SS-2 (829) and involving the host cell membrane-associated caveolin 1 (830).

Using the *in vitro* model composed of fully differentiated Caco-2-cl1 cells cocultured with the Raji B cell line, Chassaing et al. (831) have observed that AIEC LF82 bacteria interact with M-like cells and that large numbers of internalized bacteria are translocated, a phenomenon that is enhanced when the bacteria are grown in the presence of sodium cholate (832).

Transport of tEPEC occurs at similarly low levels across both native fully differentiated Caco-2 and M-like Caco-2/Raji-cocultured monolayers (828). Since translocation rates are markedly higher for tEPEC lacking either functional T3SS or the effector protein EspF, it is conceivable that T3SS effectors downregulate tEPEC translocation.

Although EHEC translocates at a low level in fully differentiated Caco-2-cl1 monolayers, it shows a high level of translocation in the *in vitro* M cell-like model composed of fully differentiated Caco-2-cl1 cells cocultured with the Raji B cell line (833).

CONCLUDING REMARKS

Our understanding of how enterovirulent bacteria cause disease by creating structural and functional dysfunctions has advanced significantly in recent years. Moreover, this increased knowledge may permit research leading to innovative antibacterial therapies. We have seen in this review that human enterovirulent bacteria have evolved several sophisticated mechanisms to subvert the host's intestinal cell machinery that result in structural and functional lesions within the human intestinal epithelial barrier. Advances in the understanding of the virulence mechanisms of enteric bacterial pathogens have been made using fully differentiated colon carcinoma cells in culture; however, it should not be forgotten that the colon cancer cell lines, subpopulations, and clone cells have several drawbacks. Although they physically and functionally mimic a human intestinal barrier in culture, they cannot be regarded as "normal" since they originate from intestinal cancer cells. Moreover, these cell lines, subpopulations, and clones also display grossly altered ploidy (834–836) and may have changes in several cellular signaling pathways as a consequence of the cancerous nature of the parental cells. For example, the cell signaling-controlled shedding of intestinal polarized epithelial cells that occurs *in vivo* during the renewal of the intestinal epithelium has never been observed in fully differentiated colon cancer cells forming a monolayer. Only a recent report describes the extrusion of cells from a fully differentiated T84 cell monolayer induced by the caspase-1-dependent action of nigericine (837). It is noteworthy that the extrusion of cells from fully differentiated colon cancer cell monolayers by enterovirulent bacteria, which is known to induce caspase-dependent cell death, currently has not been documented. In addition, it should be noted that it is difficult to carry out efficient DNA/small interfering RNA (siRNA) transfections in fully differentiated colon cancer cells. Indeed, it is difficult to achieve the complete silencing of the genes of interest in these cells by means of siRNA transfection (88). Attempts have been made to carry out transfections with some of these cell lines by transfecting the cells at an undifferentiated stage and then subculturing them to obtain transfected, postconfluent, fully differentiated cells. In many cases, these attempts have been unsatisfactory, since the functional cell differentiation process has been found to have been altered. Moreover, the difficulty of transfecting cDNA into these cell lines has led researchers to use cells that are more permissive to transfection in order to study the role of a host cell protein targeted by a bacterial virulence factor. Convincing experiments have been conducted using the transfection-permissive HeLa and Chinese hamster ovary epithelial cells or fibroblast NIH 3T3 cells.

Following experiments with cultured human epithelial cells, dissecting the different mechanisms of microbial pathogenesis requires the identification of relevant animal models of enteric infection that fulfill some criteria, including use of a host-adapted pathogen and well-defined clinical/pathological parameters that serve as predictable measures of disease. The most successful examples are the animal-pathogenic EPEC- or EHEC-like *C. rodentium* and RDEC-1 strains inducing A/E lesions at the brush border of enterocytes. However, due to highest human specificity, the pathogenesis of several enterovirulent bacteria, such as Afa/$Dr_{DAF/CEACAMs}$ DAEC, could not be investigated in infectious animal models. Human intestinal *in vitro* organ culture (IVOC) represents a valuable tool for investigation of the mechanisms of virulence of enterovirulent bacteria in an *ex vivo* human situation. Moreover, IVOCs are important in particular for examining the mechanisms of pathogenesis developed by human enteric bacterial and viral pathogens showing highest human specificity. Various IVOC systems of human intestinal cells have been established. In a recent and complete review, Fang et al. (838) have examined the different IVOC systems that have been developed and in particular those that constitute a polarized IVOC providing apical exposure to simulate an *ex vivo* infection route closely mimicking the *in vivo* infection route that occurs in the human intestine. Phillips and coworkers have established an IVOC from pediatric biopsy specimens of duodenum mounted in a modified Micro-Snapwell system specifically adapted for use with 2- to 3-mm-diameter biopsy samples. This IVOC system has been used mainly to study the mechanisms of virulence of ETEC (839), EPEC (840–851), EHEC (852–857), EAEC (507, 858), and *Salmonella* (392) and the action of Stx toxins (641). Other IVOCs have been used for studies on *C. jejuni* (859), EPEC (692, 860, 861), EAEC (645, 862–864), and *Shigella* (865) pathogenesis. Moreover, IVOCs from colonic biopsy specimens from CD patients have been used to show the adhesion of AIEC (444, 866). Recently, other IVOCs have also been described, composed of specimens of human colon taken downstream of the tumor, allowing the study of proinflammatory colonic immune responses in a context of depletion of IL-10, which regulates a complex ecosystem composed of bacteria, the intestinal epithelial barrier, and resident colitogenic cells (867, 868). Specimens of mucosa and submucosa, either cocultured or cultured alone, have been used to investigate the effect of *Clostridium difficile* toxin B on IL-8 production via the IL-1β-dependent pathway (869) and the activation of the VIPergic neurons (870). Recently, human tissue preparations consisting of separated serosa and circular muscle have been used to culture the mucosal and submucosal fragments. From this model, and given that the enteric nervous system is a potent modulator of intestinal epithelial barrier functions (30), knowledge of the role of enteric neurons in intestinal *Shigella* pathogenesis is beginning to emerge (655, 871, 872). Moreover, two coculture models, consisting of human submucosa containing the submucosal neuronal network and human fully differentiated, mucus-secreting HT29-Cl.16E or enterocyte-like Caco-2 monolayers, have been described. These models have been used to investigate the effects of submucosal neuron activation by electrical field stimulation on cell proliferation (239) and on the VIPergic neuronal pathway controlling the paracellular permeability and structural organization of TJs (70) and the PKA-independent and MAPK-dependent IL-8 production (240). In addition, an interesting *in vitro* three-dimensional organoid culture using intestinal stem cells has also been developed (873, 874).

The interest of IVOCs is that they provide an entire intestinal system comprising the four major phenotypes of cells lining the intestinal epithelial barrier. The interest of cultured human intes-

tinal fully differentiated colon cancer cell lines, subpopulations, and clone cells is that they provide useful models of each of the intestinal cell phenotypes. Experiments combining a human IVOC and a cultured human intestinal fully differentiated colon cancer cell line, subpopulation, or clone cells provide the most reliable and convincing insights into the roles of particular virulence factors in the virulence mechanisms of human enterovirulent bacteria. However, IOVCs are difficult to obtain for laboratory experimentation, because they require a close link between the research laboratory and a hospital department of gastrointestinal tract surgery and surgical pathology department. In addition, IOVCs can be used only for a limited period in culture and therefore seem to be more appropriate for investigating events that occur soon after infection in normal intestinal cells than for investigating later cellular events.

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